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STUDIES ON OXIDATION AND REDUCTION BY PNEUMOCOCCUS.

VI. The Oxidation of Enzymes in Sterile Extracts of Pneumococcus.

By JAMES M. NEILL, Ph.D., and OSWALD T. AVERY, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 16, 1924.)

INTRODUCTION.

Avery and Cullen (1, 2) have demonstrated that the pneumococcus cell contains the hydrolyzing enzymes (peptonase, lipase, sucrase, amylase, inulase) and a bacteriolytic enzyme. Cole (3) (1912), (4) (1914), has shown that pneumococci also possess a hemotoxin. These enzymes and the hemotoxin are endocellular, representing actual constituents of the cell, and consequently are demonstrable in sterile extracts prepared by disruption of the cell walls. In addition to these intracellular substances, the pneumococcus cell contains a number of oxidation-reduction systems. As shown in preceding papers, sterile extracts of pneumococci may be prepared in such a manner that some at least of these oxidation systems are preserved in an active state. Among the oxidation-reduction activities of these extracts already reported are the prompt formation of peroxide when the extracts are exposed to air (5), the consumption of molecular oxygen (6), the active reduction of methylene blue (7), and the rapid oxidation of hemotoxin (8) and of hemoglobin (6). The cell extracts are sterile; and all of these reactions are exhibited under conditions excluding the participation of living or intact cells.

In preceding papers it has been shown that the marked oxidation reactions which can be induced in these extracts are not without effect upon certain labile constituents of the pneumococcus cell. For instance, the hemotoxin, although itself non-reactive with molecular oxygen, is rapidly destroyed by oxidizing agents formed during the oxidation of other easily oxidized constituents of the extract. It seemed of considerable interest, therefore, to determine whether the hydrolyzing enzymes of Pneumococcus are destroyed in a manner analogous to that of the oxidation of the hemotoxin.

A number of examples of the inactivation of enzymes by oxidation have been reported in the literature. Sigmund (9) (1905) compared the effect of ozonized oxygen and pure oxygen upon the activity of a number of enzymes by passing the gases through enzyme solutions for 1 hour before adding the enzymes to their substrates. Treatment with pure oxygen frequently had some effect, but in all cases the ozone treatment caused a greater loss in enzyme activity. Starch-splitting enzymes and emulsin were destroyed to a significant degree, and pepsin also suffered some loss in activity. The effect upon sucrase was especially pronounced, dilute solutions of this enzyme being almost entirely destroyed. Kastle (10) (1906) has reported the destruction of lipase by ozone. Buchner and Hoffmann (11) have found that zymase and apparently also yeast sucrase are destroyed by ozone.

Hydrogen peroxide has been the oxidizing agent most frequently employed in studies of enzyme inactivation by oxidation. That catalase itself is destroyed by large amounts of hydrogen peroxide has been generally recognized since the time of the discovery of this enzyme (Senter (12), Vandevelde (13), and Vandevelde, Schoenfeld, and Leboucq, 1901 (14), and others). This occurrence is probably one of the factors which obscure the dynamics of the action of this enzyme. Peroxidase is also said to be susceptible to large amounts of hydrogen peroxide (Bach and Chodat, 1903 (15); Schönbein (16)), and to be destroyed by organic peroxides formed during the autoxidation of organic compounds (Bach and Chodat, 1903 (15); Bach, 1905 (17)). Hydrolyzing enzymes may be destroyed by hydrogen peroxide, although differences exist in the degree to which different enzymes of this type are inactivated by this chemical oxidizing agent. Vandevelde (1904) found that hydrogen peroxide in concentrations of 0.05 per cent destroyed most of the activity of starch-hydrolyzing enzymes and that concentrations of 0.2 to 0.5 per cent completely inactivated them. On the other hand, this author found that trypsin and pepsin were not destroyed and, indeed, concluded that the action of these enzymes was accelerated by the peroxide. Walbum (1911) (18) observed somewhat similar relations. Concentrations of 0.1 per cent completely destroyed ptyalin in 30 minutes, although very small amounts of hydrogen peroxide (up to 0.005 per cent) were without effect. Exposure of rennet to 0.5 per cent hydrogen peroxide for $\frac{1}{2}$ hour completely inactivated this enzyme, but pepsin and trypsin were not affected in the concentrations tested.

A number of other oxidizing agents of various natures have been found to inactivate enzymes (Senter, 1905 (19); Kastle, 1906 (10); see also review by Euler (20)). The fact observed by Falk, McGuire, and Blount (21) that a greater loss of enzyme activity occurred in vegetables dehydrated in the air than if the dehydration were done *in vacuo* is probably another example of destruction of enzymes by oxidation.

It is evident from the above review that the activity of enzymes may be destroyed by oxidation and that differences exist in the susceptibility of various enzymes to oxidation. It is also significant

that, in most instances, the destruction of the enzymes has been due not to the action of molecular oxygen, but to oxidizing agents of which peroxides have been the most studied. The literature thus furnishes evidence that enzymes of cellular origin may be destroyed by oxidizing agents *in vitro*, and indeed, by oxidizing agents similar in nature to those which are formed during the oxidation of the "complete system" type of pneumococcus extracts. In none of the experiments in the literature, however, have the oxidizing agents used in enzyme destruction been derived from cellular oxidations.

Sterile pneumococcus extracts contain not only hydrolyzing enzymes, but also the oxidation-reduction systems which form peroxides when exposed to the air. This set of circumstances afforded an opportunity to demonstrate the destruction of enzymes by oxidizing agents actually formed by constituents of the same cell as that from which the enzymes themselves are derived.

Methods.

Bacteriological.—

Sterile Pneumococcus Extracts.—The sterile pneumococcus extracts used in this investigation were prepared in the manner described in a preceding paper (5). Two types of extract have been employed: the broth extract of unwashed cells and the phosphate solution extract of washed cells. The first type of extract contains "complete" oxidation-reduction systems which actively form peroxide when exposed to molecular oxygen and which actively reduce methylene blue. The second type of extract contains potentially active, but "incomplete" oxidation-reduction systems which form peroxide and which reduce methylene blue only if the system is "completed" by the addition of easily oxidized or autoxidizable substances which may be furnished by meat infusion or yeast extract. Because of these relations, the broth extract of unwashed cells and the phosphate solution extract of washed cells are at times referred to in the text as "complete" and "incomplete system" types of extract. Both types of extract contain approximately equal concentrations of the hemotoxin described by Cole, and of the enzymes described by Avery and Cullen.

Sterility Controls.—The sterility of all extracts has been controlled by cultural and animal tests. Unless otherwise stated, the broth extract of unwashed cells was filtered through Berkefeld candles. The phosphate solution extracts were not filtered, as filtration of this type of extract results in a great loss in the active concentration of all the enzymes. The sterility of the enzyme-substrate mixtures was controlled by cultural tests.

Chemical.—

Preparation of Substrates.—Unless otherwise stated, the substrates of the various enzymes were prepared as follows: The peptone solutions used consisted of 1 per cent solutions of Fairchild's peptone in 0.06 M phosphate solution (pH 7.8). The sucrose substrates consisted of 2 per cent solutions of sucrose in 0.06 M phosphate solution (pH 7.0). The starch, inulin, and raffinose solutions used as substrates were also prepared in phosphate solution of the same pH. The lipase substrate consisted of 2 per cent emulsions of Kahlbaum's tributyrin in 0.06 M phosphate solution (pH 7.5). All of these substrates were sterilized in the autoclave for 10 minutes at 120°C. The hemotoxin titrations were performed by the procedure previously described, with 2.5 cc. of a 1 per cent suspension of washed erythrocytes from sterile defibrinated rabbit blood.

Quantitative Analyses.—The hydrolysis of peptone was determined by amino nitrogen analyses by Van Slyke's method (22). The hydrolysis of tributyrin was determined by titration of the butyric acid produced; 5 cc. of the enzyme-substrate mixture were titrated to the original pH as determined by colorimetric comparison with the control; a micro burette was used. Qualitative tests of the hydrolysis of sucrose were carried out by the Benedict test; 0.4 cc. of enzyme-substrate mixture was overlaid on 3 cc. of Benedict's solution and boiled for 3 minutes. Quantitative estimations of reducing sugars were made by Benedict's volumetric method.

Oxidation of Extracts by "Exposure to the Air."—Although constant agitation of the extract seemed desirable for experiments on the rate of destruction of the enzymes by oxidation, it seemed necessary to exclude any possibility of the so called "*Schüttel-inactivierung*" as a factor in the observed enzyme destruction. For this reason, none of the extracts were shaken during oxidation. Oxidation was accomplished by exposing the extract to the air in shallow layers (less than 1.0 mm.) in Erlenmeyer flasks. During exposure to the air, the extracts were protected from light by wrapping the flask in black paper.

The extracts themselves were stored in the ice box at 2°C. under heavy vaseline seal. At the time of their use in the various experiments, the "age" of the extracts varied from 1 to 4 weeks. Stored under these conditions, no significant losses in activity of any of the enzymes were observed.

Demonstration of Destruction of Enzymes.—In the following tests, differences in concentration of active enzyme have been estimated by differences in the amounts of products of enzyme action over certain periods of time. Most of the experiments involved determinations of total destruction of enzyme activity, which made necessary the use of large amounts of enzyme solution and long digestion periods. The methods used, therefore, while theoretically not well adapted to estimations of the actual concentrations of active enzyme, serve the purpose desired in showing relative differences in the amount of enzyme which remained active after different oxidation and heating treatments.

Influence of Oxidation upon the Subsequent Activity of Pneumococcus Sucrase.

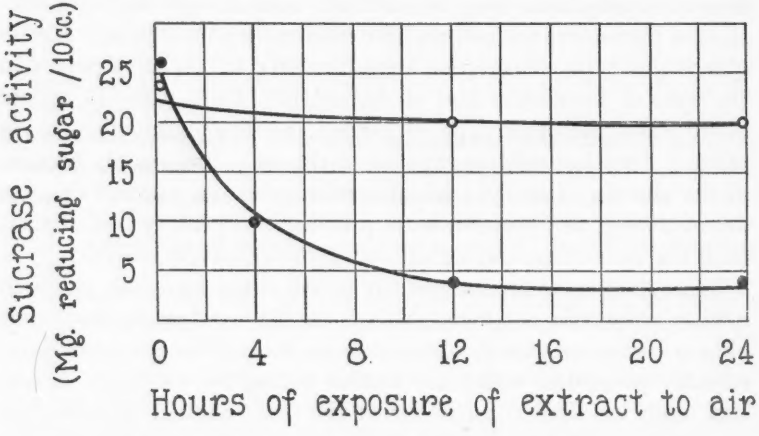
It may be recalled that when a broth extract of unwashed pneumococci is exposed to air, marked oxidative reactions occur; certain constituents of the extract are rapidly oxidized with the formation of detectable amounts of peroxide. During these oxidations, and probably through the agency of products formed by the union of molecular oxygen with the more reactive constituents of the extract, certain other substances may be oxidized, even though the latter substances themselves are non-reactive with molecular oxygen. Examples of this type of oxidation were furnished in preceding papers in the case of hemotoxin and of hemoglobin. Quite different results occur when the other type of extract, *i.e.* the phosphate solution extract of washed cells, is exposed to the air. Presumably because of the absence of easily oxidizable substances, no marked changes are evidenced and peroxide is not produced. In this type of extract, both the hemotoxin and hemoglobin prove relatively stable.

These differences are interpreted as due to the fact that, although neither hemotoxin nor hemoglobin is readily oxidized by molecular oxygen, both are rapidly destroyed by the oxidizing agents (presumably peroxides) which are formed during the oxidation of certain easily oxidized constituents of the broth extract of unwashed pneumococci. It now seemed of interest to determine whether the destruction of pneumococcus sucrase represented a similar type of oxidation. Sucrase, like hemotoxin, is contained in about equal concentrations in both types of pneumococcus extracts. Therefore, a comparison of the effect upon sucrase activity induced by aerating the two types of extract offers a means of investigating the nature of the oxidation reaction by which this enzyme is destroyed.

Accordingly, phosphate solution extract of washed pneumococci ("incomplete system" type of extract) and broth extract of unwashed pneumococci ("complete system" type of extract) were exposed to air at 37°C. After different periods of exposure, portions of the two extracts were removed and tested for sucrase activity. As controls on the effect of temperature itself upon the stability of the sucrase, portions of the two types of extract were held at 37°C., protected from air by a heavy vaseline seal, and added to the substrate at the same time as the oxidized extract.

A number of experiments of this type have been conducted. In all instances, the sucrase proved relatively stable when exposed to air in the washed cell extracts. The degree of destruction of the sucrase when the "complete system" type of extract is exposed to air varies with the initial sucrase activity and with the oxidizing (peroxide-forming) power of various extracts.

It is evident from these results (Text-fig. 1) that a rapid destruction of sucrase occurs during the oxidation of broth extracts of un-



° Phosphate solution extract of washed pneumococci.
• Broth extract of unwashed pneumococci.

TEXT-FIG. 1. Effect of exposure to air upon the subsequent sucrase activity of extracts of washed and unwashed pneumococci.

washed pneumococci. On the other hand, in the extract of washed cells, sucrase proves as stable in the presence as in the absence of air. These relations are analogous to those previously found in the study of the oxidation of hemotoxin and of hemoglobin by pneumococcus extracts. Hence, it would seem that sucrase destruction represents a similar type of oxidation. In the broth extract of unwashed cells, the destruction of the sucrase when the extract is exposed to oxygen may be assigned to oxidizing agents which are formed as products of the action of molecular oxygen upon other easily oxidized con-

stituents of the extract. In the case of the phosphate solution extract of washed cells, the stability of the enzyme seems to be explained by the fact that sucrase itself does not react readily with molecular oxygen; and that the "incomplete system" extract, unlike the unwashed cell extract, contains no easily oxidizable substances to form oxidizing agents by union with molecular oxygen.

Influence of Oxidation upon the Subsequent Activity of Other Carbohydrate-Hydrolyzing Enzymes of Pneumococcus.

It seemed probable that the pneumococcus enzymes which hydrolyze other carbohydrates might exhibit reactions similar to those

TABLE I.

Effect of Exposure to Air upon the Subsequent Activity of Pneumococcus Amylase, Raffinase, and Inulase.

Type of extract.		Enzyme activity of pneumococcus extract.		
		Amylase.	Raffinase.	Inulase.
Unwashed cells extracted in broth (complete system).	Reduced.*	+†	+++	+
	Oxidized.	−	+	−
Washed cells extracted in phosphate solution (incomplete system).	Reduced.	+	+++	+
	Oxidized.	+	+++	+

* Reduced extract stored under seal to protect from air for 24 hours at 37°C. Oxidized extract exposed to air in thin layer for 24 hours at 37°C.

† The concentration of reducing sugars represented by these symbols approximate the following values: −, less than 0.03 per cent; +, 0.10 per cent; +++, 0.30 per cent.

by which sucrase is destroyed by oxidation. To investigate this question, experiments similar to those previously described for invertase were carried out with pneumococcus inulase, amylase, and raffinase.

It is evident from the results of this experiment (Table I) that the active concentration of pneumococcus amylase, inulase, and raffinase is markedly decreased during the oxidation of the broth extract of unwashed pneumococci. Moreover, as previously observed in the case of sucrase, these carbohydrate-hydrolyzing enzymes are not

themselves reactive with molecular oxygen, since they proved stable during the exposure to air of the phosphate solution extract of washed cells.

The Effect of Oxidation upon the Subsequent Activity of Pneumococcus Lipase.

Avery and Cullen (1) have described the properties of the lipase contained in the pneumococcus cell. This enzyme was shown to split tributyrin with the production of large amounts of butyric acid. The literature reviewed presents examples of the oxidation of certain lipases by treatment with chemical oxidizing agents. Lipases from various organisms may exhibit strikingly different thermostability. In view of the known differences in resistance of various enzymes to chemical oxidizing agents, together with the variations in heat resistance of different lipolytic enzymes, it was determined to investigate the resistance of pneumococcus lipase to the oxidizing agents formed during the oxidation of pneumococcus extracts.

From the results of several experiments it is evident that the lipase activity of pneumococcus extract is not appreciably changed during the oxidation processes which occur when the "complete system" type of extract is exposed to the air. Although a series of tests involving different amounts of extract have been employed, no distinctions in the lipase action of "reduced" and "oxidized" extracts have been detected.

The results of the above experiments do not constitute evidence that pneumococcus lipase cannot be inactivated by oxidation, as subsequent experiments will reveal. However, it is obvious from them that oxidation processes which cause marked diminution in the active concentration of the sucrase, amylase, inulase, and raffinase do not have a comparable effect upon the activity of the lipase.

Influence of Oxidation of Extracts upon the Subsequent Activity of Pneumococcus "Peptonase."

Avery and Cullen (1) have shown that the pneumococcus cell contains certain constituents which hydrolyze intact proteins to some extent and split to a marked degree commercial "peptone" into simpler peptides and amino acids. As the maximum activity was exhibited in the further hydrolysis of peptide nitrogen, they applied the term "peptonase" to the enzyme (or enzymes) responsible for this proteolytic activity of pneumococcus.

From the literature reviewed, it is evident that enzymes which probably are more or less similar in nature to pneumococcus "peptonase," such as trypsin, have proved quite resistant to oxidizing agents. There are evident discrepancies in the reports of the various investigators upon the question of whether or not these enzymes are destroyed by oxidation at all, due probably to differences in experimental conditions. Evidently, however, these enzymes are among those most resistant to oxidation.

A number of experiments have been carried out to test the effect of the oxidizing agents formed in pneumococcus extracts upon the "peptonase" activity. In no instance did the oxidation of the broth extract of Pneumococcus have any detectable effect upon the activity of the "peptonase." Thus, the experiments presented have shown that the lipase and peptonase of pneumococcus are unaffected by exposure to concentrations of oxidizing agents which seriously diminish the active concentration of pneumococcus sucrase, amylase, raffinase, and inulase.

Effect of Oxidation of Extracts upon the Bacteriolytic Enzyme of Pneumococcus.

Avery and Cullen (2) have reported the presence of a bacteriolytic enzyme in Pneumococcus, which exhibits to a striking degree the property of causing lysis of pneumococcus cells which have been killed by heat, and to a less extent lysis of closely related bacteria as the *Streptococcus viridans* group. Since practically nothing is known of the nature or even of the identity of this enzyme, it seemed particularly desirable to compare its relative resistance to oxidation with that of other pneumococcus enzymes. Accordingly, comparisons have been made of the bacteriolytic activity of a broth extract of Pneumococcus which had been oxidized, with the bacteriolytic activity of an extract which had not been exposed to oxidation.

The results of these experiments cannot be given definite quantitative expression. However, repeated experiments of this nature leave no doubt of the susceptibility of the bacteriolytic enzyme to oxidation products formed during the oxidation of the "complete system" type of pneumococcus extract. In every instance, with dilutions of extract from 0.1 to 0.002 cc., the "reduced" broth ex-

tract manifested a greater bacteriolytic effect than did the same dilution of "oxidized" extract. Although differences in the activity of each corresponding dilution of "reduced" and "oxidized" extract were definite and unmistakable, the differences in no case (not even in dilutions of only 0.002 cc. of extract) approached the distinction between positive and negative bacteriolysis.

It is interesting to note the distinction between the bacteriolytic and hemolytic agents in pneumococcus extracts, which is afforded by the differences in resistance to oxidation. Although oxidation of the unwashed cell extract effects only a slight loss in the bacteriolytic activity, the hemolytic activity of the extract is completely destroyed under the same conditions.

The definite destruction of bacteriolytic activity which occurs during the oxidation of the "complete system" type of extract is manifested not at all when the phosphate solution extract of washed cells is exposed to the air. Hence, it seems that the bacteriolytic enzyme is oxidized by a reaction similar in type to those which effect the destruction of hemotoxin, sucrase, amylase, inulase, and raffinase.

The above experiments on the effect of oxidation of sterile pneumococcus extracts upon the activity of the various enzymes have demonstrated marked differences in their susceptibility to oxidation. Their resistance to the oxidation products formed when unwashed cell extracts are exposed to the air varies in the following order; peptonase, lipase, bacteriolytic enzyme, sucrase and other carbohydrate-hydrolyzing enzymes, and hemotoxin; the activity of the peptonase and lipase apparently is unaffected by oxidation reactions which seriously reduce the active concentration of other labile, pneumococcus cell constituents.

Relative Order of Heat Resistance of the Different Enzymes of Pneumococcus.

As a means of comparing the relative degree of heat resistance of the different enzymes with the relative order of their susceptibility to oxidation, tests were made of the stability of the enzymes at different temperatures, with the same pneumococcus extracts as those employed in the preceding experiments on oxidation. The degree of temperature, length of exposure, and pH of the solution are related

factors in the destruction of enzymes by heat. In these experiments the reaction of the extract (pH 7.1) and an exposure of 10 minutes were chosen as constant factors, and the degree of temperature alone was varied.

The different enzymes of Pneumococcus exhibit marked differences in heat resistance. The comparative rates of destruction of the enzymes at the different temperatures are reflected in Text-fig. 2. It is evident that those enzymes which proved more resistant to oxidation in the extract are also more resistant to heat. Thus, the known hydrolyzing enzymes of pneumococcus can be arranged in the same order of relative resistance, *i.e.* peptonase, lipase, sucrase, both from the standpoint of their resistance to heat and their resistance to the oxidation products formed during the oxidation of a broth extract of unwashed pneumococci.

Relative Resistance of Different Enzymes of Pneumococcus to the Action of Hydrogen Peroxide.

Hydrogen peroxide has been the oxidizing agent most frequently employed in studies of the destruction of enzymes by oxidation. It is evident from the literature that differences exist in the susceptibility of various enzymes to this reagent. The experiments presented in this paper make it equally evident that differences exist in the susceptibility of various enzymes of Pneumococcusto the action of the oxidizing agents which are formed during oxidation of the "complete system" type of sterile extract. If the destruction of pneumococcus enzymes under these conditions is due to the action of peroxides formed during the oxidation of pneumococcus extract, it might be expected that those enzymes the activity of which is least affected by the oxidation of the extracts, would prove most resistant to the action of reagent hydrogen peroxide.

Experiments were designed to ascertain if the order of susceptibility of the different enzymes is the same when treated with preformed hydrogen peroxide as when the enzymes are exposed to the oxidation products of pneumococcus extracts. It also seemed desirable to compare the relative susceptibility of the different enzymes to hydrogen peroxide with the relative heat susceptibility of the enzymes. With this in mind, in order that the results might be directly comparable with those already obtained in the preceding experiment, the present experiment was conducted with the same pneumococcus extract and with the same substrate preparations.

3.0 cc. quantities of the pneumococcus extract used in the preceding experiment were added to tubes containing 3.0 cc. of titrated "Dioxogen" diluted in sterile 0.06 M phosphate solution (pH 7.2). The final concentration of H_2O_2 in the extract-H_2O_2 mixtures was 150, 30, and 6 mM (approximately 0.5, 0.1, and 0.02 per cent H_2O_2). The tubes were sealed with vaseline and placed in a water bath for 24 hours at 37°C. The peroxide-treated extract, and untreated extract, were then added to the substrate mixtures to give the same concentration of pneumococcus extract as those used in the preceding experiment.

The results of this experiment (Table II) demonstrate that the enzymes of Pneumococcus differ in their relative susceptibility to the action of hydrogen peroxide. Furthermore, the peptonase and lipase enzymes which were unaffected by the oxidation products formed in

TABLE II.

Relative Resistance of Different Enzymes of Pneumococcus to the Action of Hydrogen Peroxide.

H_2O_2 to which enzyme had previously been exposed.	Peptonase.		Lipase.		Sucrase.	Bacteriolytic enzyme.	Hemotoxin.
	NH_2-N in 100 cc.		pH	N/14 acid in 10 cc.	Reducing sugar in 100 cc.	Bacteriolysis by 0.02 cc. extract.	Hemolysis by 0.10 cc. extract.
	Total.	Increase.					
mM	*mg.*	*mg.*		*cc.*	*mg.*		
0	65.1	12.1	5.7	4.10	26	++++	++++
6	57.9	4.9	6.8	2.0	3*	±	–
30	54.8	1.8	7.5	–	–	–	–
150	52.9	–	7.5	–	–	–	–

* Estimated by comparison with standard glucose solutions.

sterile pneumococcus extracts proved most resistant to the action of reagent hydrogen peroxide. The relative order of resistance to H_2O_2 exhibited by the peptonase, lipase, and sucrase of Pneumococcus also agrees with the order of heat resistance of the same enzymes. The lipase and peptonase enzymes of Pneumococcus in the above experiment are inactivated by considerably less drastic treatment with H_2O_2 than would appear from the literature to be the case with protein-splitting and lipase enzymes derived from higher organisms. Possibly the pneumococcus lipase and peptonase represent enzymes different in nature from the lipases and protein-splitting enzymes more commonly studied, as these pneumococcus enzymes are less resistant both to heat and to oxidation.

The Selective Destruction of Pneumococcus Enzymes by Oxidation and by Heat.

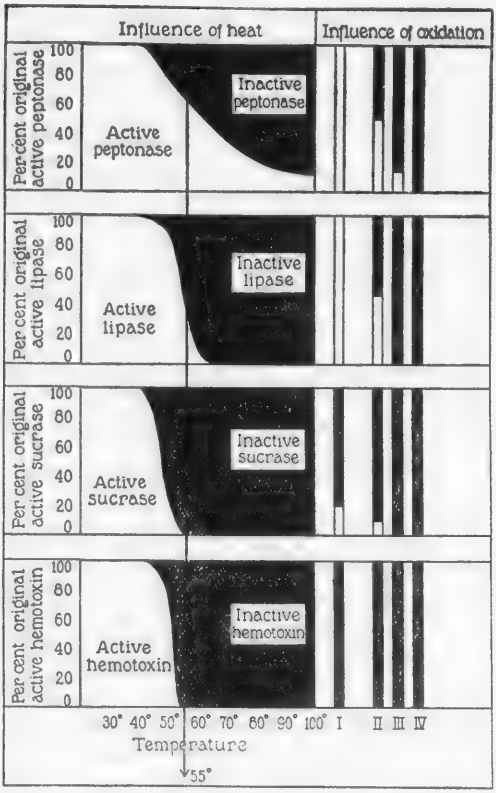
In a study of biochemical processes, mixtures of the constituents of certain cells are frequently employed. In such cases, the assignment of definite functions to the activity of a particular constituent demands the elimination or inactivation of other active substances present. One of the most frequent methods employed for this purpose is the "fractional inactivation" of the various constituents by heating at various temperatures. By a proper choice of temperatures, the more heat-stable constituents of the mixture may be retained in an active form, and the more heat-labile substances may be destroyed. In many cases, however, this method is limited by the fact that the fractionation is by no means sharp; temperatures which suffice for the complete inactivation of the more labile substances also effect serious loss in the active concentration of the relatively heat-stable constituents.

Oxidation of a mixture of biochemically active substances offers a further means of separation of certain of the active constituents. Recently, it has been shown that oxidation of cod liver oil results in the complete destruction of the fat-soluble vitamine A without any inactivation of the antirachitic vitamine (23). Inspection of data already presented in this paper reveals the fact that oxidation will also serve as a means of separating certain of the active constituents of a mixture of pneumococcus cellular substances. By properly regulated oxidation, the "fractional inactivation" of these various constituents is apparently quite as sharp as can be obtained by heating the extract at any temperature. To illustrate these relations, data of the preceding experiments are presented graphically in Text-fig. 2.

In this figure, there is plotted the relative amount of each of the original enzymes[1] which remains active when a mixture of pneumococcus cellular substances is heated at various temperatures or is exposed to

[1] The assumption is not made that pneumococcus hemotoxin is an enzyme. It is included in this study for purposes of comparison merely, as a known labile constituent of the pneumococcus cell.

The bacteriolytic enzyme is not included in this figure, due to the difficulty of obtaining a quantitative expression of the action of this enzyme.



TEXT-FIG. 2. Comparison of the influence of heating at different temperatures and the influence of oxidation, upon the subsequent activity of different enzymes of Pneumococcus.

The relative amount of the various enzymes which remains active after heating or after oxidation is calculated from data obtained in preceding experiments in this paper. (The data for the effect of heat upon hemotoxin are taken from Table II in a preceding paper (8).) It is assumed that the products produced by the untreated or original enzyme solution represent 100 per cent of active enzyme. The fact that this assumption is not theoretically correct (see under Methods) influences the slopes of the various curves, but does not affect in any way the interpretation given in the text.

The Roman numerals in this figure refer to the following: I, oxidized broth extracts of unwashed pneumococci; II, exposure for 24 hours to 6 mM H_2O_2 ("Dioxogen"); III, exposure for 24 hours to 30 mM H_2O_2 ("Dioxogen"); and IV, exposure for 24 hours to 150 mM H_2O_2 ("Dioxogen").

differing concentrations of oxidizing agents. The unshaded area in each case represents the percentage of original enzyme which is still active after heating or after oxidation; the shaded area represents the portion of the originally active enzyme which has been inactivated. A vertical line drawn from any point on the temperature axis is an indication of the percentage inactivation of the various enzymes after exposure for 10 minutes at that temperature. Analysis of the curves of the effect of temperature upon the activity of the several enzymes reveals the fact that a "fractional inactivation" by 10 minutes heating at different temperatures would by no means be distinct. Exposure of an extract for 10 minutes to 55°C., which suffices for the inactivation of hemotoxin and of sucrase (the most labile constituents of the mixture), results in a serious loss in activity of the more heat-resistant lipase and peptonase. On the other hand, it seems possible that, by the proper choice of oxidizing agents in regulated concentrations, some at least of the more easily oxidized constituents can be completely inactivated without a serious loss in activity of certain other constituents. This is evident in the case of the broth extract of unwashed cells which has been oxidized by exposure to air at 37°C. (I in Text-fig. 2). Owing to fortuitous conditions during the oxidation of these extracts, oxidizing agents are produced in concentrations sufficient to destroy all of the hemotoxin and most of the sucrase. Nevertheless, in spite of this degree of oxidation, the hemotoxin-free and sucrase-poor extract still manifests its original peptonase and lipase activity. The experiments with different concentrations of hydrogen peroxide did not furnish as sharp distinctions. Possibly analogous distinctions between these enzymes might be obtained by the requisite concentrations of this oxidizing agent, as later experiments have shown that certain more easily oxidized pneumococcus cell constituents are inactivated by concentrations of hydrogen peroxide which are without effect upon the above mentioned enzymes of Pneumococcus.

DISCUSSION.

The sterile bacterial extracts used in this series of studies have been prepared by freezing and thawing anaerobically grown pneumococci. Owing to the method of preparation, the extracts contain all

of the intracellular constituents of Pneumococcus which are liberated by rupture of the cell wall. These extracts, therefore, include not only the oxidation-reduction systems reported in the immediately preceding papers, but also the endocellular hemotoxin and enzymes previously reported from this laboratory.

The literature furnishes evidence that enzymes may be destroyed, *in vitro*, by oxidizing agents similar in nature to those which are formed during the oxidation of broth extracts of unwashed pneumococcus cells. Sterile pneumococcus extracts represent systems containing not only a number of well known cellular enzymes but also reactive substances which form oxidizing agents when exposed to molecular oxygen. There was at hand, therefore, an opportunity to study the destruction of enzymes by oxidizing agents actually formed by constituents of the same cell from which the enzymes have been derived. That oxidizing agents formed by sterile extracts of pneumococci can oxidize hemotoxin, one of the labile cellular substances, has already been reported (Avery and Neill). The present investigation was designed with the object of furnishing examples of the fact that oxidizing agents (presumably of peroxide nature) may be formed from molecular oxygen by bacterial cells, and that these oxidizing substances may in turn destroy other labile, but important cell constituents.

The results of the present investigation reveal the fact that the oxidizing agents formed when the cellular substances are exposed to air do exert a destructive action upon certain of the enzymes of Pneumococcus. Differences are manifest in the resistance of the various enzymes to these oxidation reactions. The carbohydrate-hydrolyzing enzymes (sucrase, amylase, inulase, raffinase) prove the most susceptible to inactivation by oxidation. The bacteriolytic enzyme also suffers a loss in activity, while the peptonase and lipase are more resistant.

Enzymes of Pneumococcus which prove stable when exposed to the air in phosphate solution extracts of washed cells are oxidized during the oxidation of broth extracts of unwashed cells. Apparently, therefore, these enzymes are inactivated by oxidation reactions similar to those previously observed in the case of hemotoxin and hemoglobin. The enzymes seem to be relatively unaffected by molecular

oxygen itself, although they are oxidized by oxidizing agents formed by the action of molecular oxygen upon certain other, easily oxidized substances in the extract. The type of reaction may be represented as follows:

C represents the labile cellular constituent of a pneumococcus oxidation-reduction system; RH, a thermostable substance easily oxidized in the presence of C. It is evident from an analysis of preceding work that C is apparently present in both the "complete" and "incomplete" types of extract, while RH is apparently absent in the latter; that is, from the extract of washed cells.

(1) [C + RH] + sucrase + O_2 → ROOH + sucrase → inactivated sucrase.
(2) [C] + sucrase + O_2 → sucrase not inactivated.

Whether the oxidizing agent is actually a peroxide cannot be demonstrated definitely, although this seems to be the case. In any event, the inactivation of the enzymes is due to oxygen of higher potential which is furnished during the oxidation of other substances in the same extract.

Experiments have also been made on the relative resistance of various pneumococcus enzymes to heat and to the action of reagent hydrogen peroxide. As a whole the results demonstrate that pneumococcus hydrolyzing enzymes may be arranged in the same order of relative stability, as concerns resistance to heat, to the action of hydrogen peroxide, and to the oxidizing agents formed when pneumococcus extracts are exposed to air.

SUMMARY.

1. Certain enzymes of Pneumococcus are destroyed by oxidizing agents formed when sterile extracts of the cellular substances are exposed to air. The carbohydrate-hydrolyzing enzymes (sucrase, raffinase, inulase, and amylase) are the most easily inactivated under these conditions, although the bacteriolytic enzyme is also reduced in activity. Similar treatment is without effect upon the active concentration of pneumococcus lipase and peptonase.

2. The enzymes which are destroyed during the oxidation of unwashed cell extracts are themselves non-reactive with molecular oxygen. The reactions by which they are destroyed seem to represent oxidations of a type similar to those proposed in previous papers for the oxidation of hemotoxin and of hemoglobin.

3. A study has been made of the relative resistance of different pneumococcus enzymes to heat and to the action of hydrogen peroxide.

4. The various enzymes may be arranged in the same order of relative resistance whether the rating be made from the standpoint of resistance to heat or of resistance to oxidation. Nevertheless, it appears that by a proper regulation of conditions of oxidation, certain labile constituents of a mixture of cellular enzymes may be inactivated with less effect upon the activity of other constituents of the mixture than when inactivation is brought about by heat.

CONCLUSION.

When sterile extracts of the cellular substances of Pneumococcus are exposed to air, oxidizing agents are formed which destroy many of the cell enzymes.

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STUDIES ON OXIDATION AND REDUCTION BY PNEUMOCOCCUS.

VII. Enzyme Activity of Sterile Filtrates of Aerobic and Anaerobic Cultures of Pneumococcus.

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In the preceding paper (1) it has been shown that the oxidation products formed during the oxidation of broth extracts of unwashed cells exert a deleterious action on certain enzymes of Pneumococcus. These enzymes are endocellular and are actual constituents of the pneumococcus cell. If cultures of Pneumococcus are filtered during the period of active growth before autolysis occurs, the enzymes are retained in the cells and are not demonstrable in the cell-free filtrate.

During cell autolysis which accompanies senescence of Pneumococcus the intracellular enzymes are liberated into the culture medium almost as completely as when the cells are dissolved by bile or are ruptured by freezing and thawing. Hence, it would seem that all of the intracellular enzymes which are demonstrable in bile solutions or frozen and thawed suspensions would likewise be present in the sterile culture filtrates. Avery and Cullen (2) have, in fact, demonstrated an active lipase and peptonase in the filtrates of autolyzed cultures. However, in view of the results of the preceding paper, it is evident that the enzyme activity of sterile filtrates of pneumococcus cultures will depend not only upon whether the endocellular enzymes are liberated into the culture medium, but also upon whether the enzymes have been protected from products of oxidation. Accordingly, one might expect to find much the same relations between the enzyme activity of the filtrates of anaerobic and of aerobic cultures as those already demonstrated between "reduced" and "oxidized" broth extracts of unwashed cells. The most obvious difference between the broth extracts of unwashed cells and the filtrates of autolyzed

cultures consists in the concentration of the cellular substances. However, an equally important difference exists in the concentration of the peroxide which is formed in aerobic cultures in comparison with that which accumulates in "oxidized" cell extracts. Although peroxide is formed in relatively large amounts during the oxidation of a sterile pneumococcus extract, it gradually decreases in concentration and frequently disappears within 24 hours. On the other hand, peroxide persists for long periods in aerobic broth cultures of Pneumococcus (Avery and Morgan (3)) since apparently the cells continue to respire with the production of peroxide long after active cell growth ceases (Avery and Neill (4)).

A more complete destruction of enzymes, therefore, might be expected in an aerobic, autolyzed culture than in an "oxidized" sterile cell extract, since the initial concentration of the enzymes is smaller in the unconcentrated culture fluids and since the period of exposure of the enzymes to high concentrations of peroxide is considerably longer in the case of the aerobic cultures. Accordingly, it is possible that some of the pneumococcus enzymes that are unaffected during the oxidation of the sterile cell extracts might be destroyed or at least seriously impaired by prolonged exposure to the oxidation products which accumulate in the aerobic cultures. To test these relations, comparisons have been made of the activity of various intracellular enzymes in filtrates of autolyzed cultures of pneumococci grown under aerobic and anaerobic conditions.

In unagitated culture flasks containing large volumes of broth with a small surface exposed to the air the medium has a low oxygen tension and presents reducing conditions not unlike those prevailing in anaerobic cultures. Under these circumstances the enzymes are afforded a considerable degree of protection from oxidizing agents. Therefore, in order to facilitate the ready diffusion of oxygen into the culture fluid, the aerobic cultures used in the following experiments were grown in broth with a large surface area exposed to air.

Aerobic and anaerobic cultures of four strains of pneumococci (Type I, I 93; Type II, G and D 39; Type III, A 66) were prepared by adding equal inocula of each strain to a series of flasks of plain broth. For the aerobic series, 50 cc. of broth were exposed to the air in 300 cc. Erlenmeyer flasks; in the case of the anaerobic series, 100 cc. Florence flasks were filled to the neck with broth and

sealed with vaseline after inoculation. The inoculated flasks were incubated at 37°C. for 7 days, after which time autolysis was practically complete in each culture of both series.

To control any effect of oxygen tension upon the actual elaboration of the enzymes, one strain (G), was grown in a 200 cc. anaerobic culture. At the end of 36 hours one portion of this culture was exposed to air under the above conditions and the other portion was allowed to autolyze under anaerobic conditions.

The autolyzed cultures of all the strains were then filtered through Berkefeld V candles; the anaerobic cultures were filtered in a nitrogen atmosphere by the method employed for the filtration of sterile "reduced" extracts. The filtrates were subcultured in blood media as sterility controls.

TABLE I.

Comparison of Activity of Various Enzymes in the Filtrates of Aerobic and Anaerobic Cultures of Pneumococcus.

Strain.	Lipase. N/14 acid in 10 cc.		Sucrase. Reducing sugars.		Bacteriolytic enzymes. Bacteriolysis.		Hemotoxin. Hemolysis.	
	Aerobic filtrate.	Anaerobic filtrate.	Aerobic filtrate.	Anaerobic filtrate.	Aerobic filtrate.	Anaerobic filtrate.	Aerobic filtrate.	Anaerobic filtrate.
	cc.	*cc.*						
G	0.8	5.2	−*	++†	−	+++	−	+++
I 193	1.0	4.9	−	++	‡	‡	‡	‡
D 39	0.6	4.8	−	++	−	+++	−	+++
A 66	0.8	5.0	−	++	−	+++	−	++++

* Negative test; less than 0.03 per cent glucose.

† Between 0.2 and 0.3 per cent glucose.

‡ Not determined.

The sterile filtrates of the aerobic and anaerobic cultures were tested for enzyme activity by the substrate-enzyme mixtures indicated below.

Lipase.—10 cc. filtrate + 0.2 cc. tributyrin.

Sucrase.—10 cc. filtrate + 1.0 cc. 20 per cent solution of sucrase.

Bacteriolytic Enzyme.—0.1, 0.5, and 1.0 cc. of filtrate were added to a series of tubes containing 0.5 cc. suspension of heat-killed pneumococci (Type II) in 0.10 M phosphate solution (pH 7.6); the volume was made up to the 1.5 cc. in each case with salt solution.

Hemotoxin.—0.5 cc. filtrate + 2.5 cc. of 1 per cent solution of red blood cells.

The activity of the various enzymes in the filtrates was determined by the methods used in analogous experiments in the preceding paper (1). The results are given in Table I.

The results of the type experiment given in Table I show that the enzyme activity of the filtrates of anaerobic cultures of Pneumococcus is much greater than that of the filtrates of aerobic cultures. The hemotoxin, sucrase, and bacteriolytic enzymes were not demonstrable in any of the aerobic filtrates, although all of these substances were active in the filtrates of the anaerobic cultures.

The bacteriolytic enzyme, which was destroyed only to a small extent during the oxidation of the sterile cell extracts, was completely inactivated in the autolyzed cultures which were exposed to air. Similarly, although lipase was apparently unaffected by oxidation of the extracts, this enzyme was almost completely destroyed in all of the aerobic cultures. It is evident, then, that the exposure of the enzymes to the oxidizing agents present in the aerobic cultures results in a more complete loss of activity than that which occurs during the oxidation of the sterile extracts. The destruction of the relatively resistant enzymes in the aerobic cultures is no doubt due to the longer exposure to higher concentrations of peroxide.

Differences in enzyme activity of the filtrates of aerobic and anaerobic cultures would seem to be due to three possible factors: (1) the possible influence of different oxygen tensions during cell growth upon the actual elaboration of the various enzymes and other cell constituents; (2) the possible influence of oxygen tension upon the liberation of the elaborated enzymes by autolysis; and (3) the destruction of the enzymes, after their elaboration, by oxidizing agents in a manner analogous to that occurring during the oxidation of sterile pneumococcus extracts. The first of the above factors seems to be eliminated since anaerobic cultures show a similar loss in enzyme activity when later allowed to autolyze under aerobic conditions. The influence of oxygen concentration upon autolysis and hence upon the liberation of the enzymes seems also to be eliminated, as there was no significant difference in the degree of autolysis finally reached in the aerobic and anaerobic cultures. Hence, the observed differences in the enzyme activity of the filtrates of aerobic and anaerobic cultures of Pneumococcus may be attributed to an actual destruction of the formed enzymes by oxidation reactions analogous to that which occurs during the oxidation of sterile broth extracts of unwashed pneumococci.

SUMMARY.

Sterile filtrates of autolyzed anaerobic cultures of Pneumococcus contain much higher concentrations of active endocellular enzymes than do the filtrates of autolyzed aerobic cultures. These differences in the enzyme activity of the filtrates of aerobic and anaerobic cultures may be explained by a destruction of the formed enzymes by oxidation reactions analogous to the destruction previously observed during the oxidation of sterile broth extracts of unwashed pneumococcus cells. The occurrence of more complete destruction of enzyme activity in autolyzed aerobic cultures than in "oxidized" sterile cell extracts is probably due to the longer exposure to oxidation products.

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THE INFLUENCE OF THYROIDECTOMY, GONADECTOMY, SUPRARENALECTOMY, AND SPLENECTOMY ON THE THYMUS GLAND OF RABBITS.

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Plates 17 and 18.

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Investigations of the ductless glands during the past 10 years which have required removal of the thyroid, parathyroids, thymus, spleen, suprarenals, and gonads alone, or in several combinations, have afforded us the opportunity of studying some of the effects of such insufficiencies on the remaining tissues of rabbits. The material accumulated under our immediate direction now seems sufficiently extensive to permit of certain statistical studies. In this paper we will report only the data relative to the effect of thyroidectomy, splenectomy, suprarenalectomy, gonadectomy, and combinations of these gland removals on the thymus gland as measured by its involution, persistence, or regeneration.[1] Lymphoid tissues other than the thymus are also affected and will be referred to briefly. The data of 373 protocols have been included in this study. In sorting the material, protocols have been discarded for one or another of the following reasons: (1) animals with chronic infections (except snuffles), (2) animals which survived a given operation less than 2 weeks, (3) animals for which the notes of the gross or microscopic examination of the necropsy tissues were incomplete, and (4) animals on which the operation or necropsy was performed by individuals other than the authors. Of more importance than all of these factors of elimination in determining the value of such records, of

[1] All operations were performed under ether anesthesia.

course, are the skill and experience of the operators and the personal daily after care of the animals. In all the experiments on which these studies are based two of the authors took part in the operation and subsequent necropsy, thus insuring uniformity of methods and records. It should be emphasized that in so simple a matter as surgical technique, training and experience are essential and these prerequisites cannot be acquired any more easily for an animal than for man. The literature of experimental pathology and physiology is glutted with reports based on insufficient numbers of experiments, on inadequate technical skill and facilities for the operation in question, and on a lack of experience and uniformity in the after care.

The material has been condensed into Table I, arranged according to the several glands removed and arbitrarily divided into four age groups: (1) rabbits under 7 months of age, (2) rabbits from 7 to 13 months of age, (3) rabbits from 13 to 19 months of age, and (4) rabbits over 19 months of age. The value of the age groups may be estimated from the fact that 257 of the 373 rabbits were born and reared in the laboratory (Medical Department of Western Reserve University and Laboratory Division of Montefiore Hospital). In the remaining 116 the age was estimated. The age under which each animal is listed was determined by its age at the time of the first operation. The anatomical condition of the thymus found at necropsy and checked in every instance by microscopic examination is listed under one of the four arbitrary groups. Each of these groups is illustrated in the accompanying photomicrographs. Thus, +− (Fig. 1) indicates complete involution and +++ (Fig. 4) represents the large, firm, lobulated, very cellular and fully active organ, while ++ (Fig. 3) and + (Fig. 2) represent early and moderate involution. At present one cannot tell with certainty whether a given thymus (celloidin sections stained with hematoxylin and eosin) is involuting from a more active stage or regenerating from a less active stage. It will be recalled that the same difficulty applied in the case of the thyroid gland until this shortcoming in interpreting thyroid morphology was overcome by means of iodine determinations. In many cases the appearance of the medulla and the presence or absence of mitotic figures make the distinction in the case of the thymus fairly certain.

TABLE I.

Glands removed.	No. of rabbits.	Condition of thymus.															
		Under 7 mos.				7–13 mos.				13–19 mos.				Over 19 mos.			
		+−	+	++	+++	+−	+	++	+++	+−	+	++	+++	+−	+	++	+++
Normals	122	2	6	4	27	16	18	14	9	13	6	2	2	3	0	0	0
Thyroidectomy	66	3	2	1	2	10	5	4	0	16	8	1	0	10	3	1	0
Gonadectomy	18	1	0	0	3	0	2	3	3	1	1	0	1	1	0	2	0
Suprarenalectomy	89	0	3	0	14	9	2	11	28	2	2	6	8	1	0	1	2
Thyroidectomy and suprarenalectomy	27	0	0	0	0	7	4	3	0	8	1	1	0	2	1	0	0
Gonadectomy and suprarenalectomy	15	0	0	0	0	0	0	0	8	0	0	0	3	1	0	0	3
Gonadectomy and thyroidectomy	25	0	0	0	0	2	5	3	0	4	2	0	0	4	4	1	0
" thyroidectomy, and suprarenalectomy	11	0	0	0	0	1	0	0	0	3	0	2	1	1	0	0	3

Analysis of Data.

Thymus in Normal Rabbits.—122 normal rabbits including 23 with splenectomy have been tabulated to show the normal variations in thymus involution in relation to age. The youngest rabbit was 110 and the oldest 631 days of age. The earliest age at which complete thymus involution was noted was 5 months and the oldest rabbit with active thymus was 448 days of age. Thymus involution in this series of rabbits occurred more frequently between the 7th and 9th months. It is hastened by pregnancy. Very wide variations both in the time interval between onset and completion of involution occur—a fact which in itself affords the best general proof that growth, persistence, and involution of this organ depend on the chemical interaction of many other organs. Some of the important functional interrelations with the thymus as revealed in this study will be pointed out in the sections that follow.

The Thymus in Splenectomized Rabbits.—71 of the 373 rabbits were splenectomized. Splenectomy alone was performed in 23; splenectomy plus thyroidectomy in 20; splenectomy plus gonadectomy in 7; splenectomy plus suprarenalectomy in 1; splenectomy plus gonadectomy plus thyroidectomy in 6; splenectomy plus gonadectomy plus suprarenalectomy in 2; splenectomy plus thyroidectomy plus suprarenelectomy in 8; and splenectomy plus thyroidectomy plus suprarenalectomy plus gonadectomy in 4. No definite influence on the thymus could be detected and on this account all splenectomized rabbits have been included either under normals,—when splenectomy was the only operation,—or under the associated gland when splenectomy was combined with other organ removals.

The Thymus in Thyroidectomized Rabbits.—Thyroidectomy alone was performed in 43 cases and combined with splenectomy in 23 cases. Of the 8 rabbits under 7 months of age 5 had involuted thymuses, while of 39 normal rabbits under 7 months of age only 8 had involuted thymuses. In rabbits over 7 months old the thymus is normally involuting. This fact makes it difficult to evaluate the hastening effect of thyroidectomy on thymus involution. Reference to the table, however, shows that the percentage of thyroidectomized rabbits with involuted thymuses is much greater than in normals of

corresponding age groups. No difference referable to sex was detectable. Further, it should be pointed out that it is often difficult to remove the thyroid entirely in rabbits. About 20 per cent of the rabbits of this series that survived longer than 1 month had unremoved thyroid fragments, but in spite of this involution-inhibiting factor, there is an obvious increase in the number of involuted thymuses.

One may conclude definitely that thyroidectomy in the rabbit hastens thymic involution.

Our data, therefore, confirm the observations of many other investigators, including Jeandelize (1), Jeandelize, Lucien, and Parisot (2), Hofmeister (3), Boccio (4), Hammett (5), and others. Hammett working with rats could not eliminate the possible additional influence of the parathyroids. It is possible to do this in rabbits and the results show that thymic involution occurs when the external parathyroids are intact and therefore may be quite independent of the parathyroid factor. Positive evidence of the thyroid-thymus interrelationship has also been obtained from feeding thyroid to animals. Hoskins (6) noted thymic enlargement in guinea pigs, Kahn (7) observed thymic enlargement in tadpoles, and Courrier (8) reported similar findings in white rats. Further evidence of the relationship has been furnished by Gudernatsch (9) and confirmed by Uhlenhuth (10), who observed that thymus feeding delayed and even prevented metamorphosis of frog and salamander larvæ.

No systematic studies of the effect of thymus removal on the thyroid have been made. Lucien and Parisot (11) removed thymuses in 6 rabbits, MacLennan (12) also working with rabbits and Soli (13) working with guinea pigs concluded that no positive thyroid reaction results. Dustin and Zunz (14) and Scammon (15) working with small series of human cases failed to find evidence of a thyroid-thymus interrelationship.

Pineles (16), Schilder (17), Erdheim (18), Rocaz and Cruchet (19), and others have pointed out that the thymus is atrophic in infantile myxedema associated with atrophy or absence of the thyroid. Simple goiter occurring after puberty in both man and animals is not usually associated with persistent thymus. There are many reports in the literature to the contrary but careful study suggests either that the association occurred before normal thymic involution had begun or that other factors than the involution-preventing effect of thyroid were present. The enlargements of the thymus classically present in Graves' disease, in Addison's disease, in acromegaly, and in status lymphaticus, while without doubt requiring the presence of an active thyroid, are more immediately dependent upon the partial loss of suprarenal and gonadal function, as will be pointed out later.

The Thymus after Gonadectomy.—Gonadectomy alone was performed in eighteen cases. The series, though small, clearly shows that gonadectomy delays thymic involution in both males and females.

This fact had already been established through the work of Henderson (20), Calzolari (21), Goodall (22), Gellin (23), and others (13). Regarding the important question of regeneration of the thymus following gonadectomy, our data are insufficient. That gonadectomy does tend to cause hypertrophy and regeneration of the thymus seems likely, but this stimulus in itself is too weak to do more than cause slight further enlargement (if done during the growing stage) and delay involution. Gonadectomy will not permanently prevent thymic involution. There is some evidence that thymectomy hastens slightly the onset of sexual maturity in rabbits (24, 25), but this effect is too slight and the variations are too great to be established with certainty by the methods used. The involution that sets in after sexual maturity may be hastened by pregnancy. A corresponding hastening of involution associated with increased sexual activity in the male is not so readily demonstrable.

The Thymus after Suprarenalectomy.—89 cases with bilateral suprarenalectomy (done in two stages) are included in this series. As mentioned above, only those that survived the removal of both suprarenals 15 days have been included. An outstanding fact is revealed—that only 12 of the 89 cases had involuted thymuses irrespective of the age of the rabbits at the time of removal, and in 52 of the 89 cases markedly hyperplastic thymuses were present. Thymus hyperplasia occurs after suprarenalectomy in all age groups; that is, in rabbits known to be over 19 months old, as well as in those under 7 months of age at the time of removal of the second suprarenal. There occurs, therefore, not only an arrest of involution but actual regeneration of the thymus. No difference referable to sex was made out. Involution is permanently delayed if the suprarenal insufficiency is permanent, but as surviving rabbits always have accessory interrenal glands there is a gradually increasing compensation and after months it may be sufficient to initiate thymic involution. We have, however, repeatedly seen fully active thymuses in rabbits 6 months after suprarenalectomy that with crude test of clinical behavior seemed fully compensated. This regeneration includes both the medulla and the cortex, though it begins in the medulla. The medulla seems to act as a specialized

type of germinal center somewhat analogous to the medulla of regenerating lymph nodes. The lymphoid tissues throughout the body (regional lymph nodes, intestinal, intrathyroid, and peribronchial) also undergo enlargement as pointed out by Crowe and Wislocki (26). This enlargement is of the same general nature as that of the thymus. The anatomical condition closely resembles that present in status lymphaticus. Unilateral suprarenalectomy was performed in 31 rabbits and does not ordinarily create sufficient suppression of the suprarenal function to elicit thymus hypertrophy.

Thymectomy, according to Soli (13), in chickens and guinea pigs, causes no detectable change in the suprarenals. The literature relative to experimental regeneration of the thymus after suprarenal injury is very meager and contains little positive evidence of this striking reaction. It has been reviewed by Jaffe (27) in connection with his quantitative studies on thymic regeneration in the rat.

The Thymus after Thyroidectomy Plus Suprarenalectomy.—27 rabbits are included in this series. The protocols of 15 of these were published in connection with studies on the effect of suprarenalectomy in thyroidectomized rabbits on heat production (28). This series reveals another striking fact, namely that 17 of the 27 cases had completely involuted thymuses, while in the series with suprarenalectomy alone only 12 of 89 cases had involuted thymuses. The thyroid hormone is, therefore, necessary for thymus hyperplasia which usually follows sufficient but sublethal injury of the suprarenal function. It has already been pointed out that thyroidectomy alone brings about thymus involution. In several of these cases fragments of thyroid were present at necropsy, but in spite of this, thymic regeneration was inhibited. On the other hand, 4 cases were discarded because of marked regeneration of the thyroid before suprarenalectomy and in these thymic regeneration had occurred. Thyroidectomy within a few days after suprarenalectomy was unsuccessful on account of the great mortality in attempting further operations on suprarenalectomized animals.

The Thymus in Gonadectomized and Suprarenalectomized Rabbits.—15 cases survived the removal of these glands more than 15 days. The mortality from suprarenalectomy is very much greater in gonadectomized rabbits than in normals. This, we believe, is due to

the removal, with the gonads, of accessory interrenal masses which for the most part occur in the lower part of the spermatic cord or about the upper pole of the ovary. The mortality is still greater when gonadectomy or any other operation is done after suprarenalectomy. 11 of the 15 cases which survived more than 15 days were suprarenalectomized after gonadectomy. In all but 1 case there was present at necropsy extreme regeneration of the thymus. All these rabbits were over 7 months of age before either operation was performed and 4 were over 19 months of age. While it is true we do not know the actual condition of the thymus in these rabbits before operation, we cannot doubt the occurrence of thymic regeneration, especially when the results are compared with those obtained in the other groups described. Gonadectomy alone delays thymic involution. It is too weak a stimulus to bring about regeneration. Suprarenalectomy alone not only delays involution, but usually causes regeneration. Gonadectomy and suprarenalectomy result in certain regeneration of the thymus in the rabbit with intact thyroid at any age. The combined effect of both gonadectomy and suprarenalectomy is greater than that of either of these operations alone. The combined operation in the rabbit permanently prevents thymic involution provided the suprarenal insufficiency is also permanent. Both gonadectomy and suprarenalectomy appear to exert qualitatively similar effects on the thymus, the difference being one of degree. As already pointed out the lymphoid tissue throughout the body also undergoes hypertrophy with the thymus which in some ways at least is analogous to that present normally in sexually immature animals.

The Thymus after Thyroidectomy and Gonadectomy.—25 cases are included in this group—all over 7 months of age. In 6 the spleen was also removed. Only 4 of the 25 had hyperplastic thymuses, while 12 of the 18 in which gonadectomy alone was performed had enlarged thymuses. When compared with thyroidectomy alone, thyroidectomy plus gonadectomy gives a larger percentage with hyperplastic thymuses. Gonadectomy can, therefore, offset to some extent the accelerating influence of thyroidectomy on thymus involution. The percentage with enlarged thymuses is also slightly higher than in the group with thyroidectomy and suprarenalectomy. The

reason for this apparent discrepancy, we think, is that thyroidectomy was done in most cases after gonadectomy and in several of these rabbits marked regeneration of the thyroid had occurred.

The Thymus after Thyroidectomy, Gonadectomy, and Suprarenalectomy.—11 cases are included in this group, of which 4 were also splenectomized. Thyroidectomy was performed in all cases before either gonadectomy or suprarenalectomy and a detailed analysis of the series shows that when regeneration of the thyroid occurred gonadectomy and suprarenalectomy caused thymic regeneration, thus strengthening the belief that the thyroid secretion is necessary for thymic regeneration even in the presence of the most powerful stimulus for thymic regeneration; namely, the combined effect of gonadectomy and suprarenalectomy.

DISCUSSION.

Four striking facts have been brought out by this statistical study of the condition of the thymus in 373 rabbits following the removal of other organs of internal secretion. (1) Thyroidectomy hastens involution of the thymus and other lymphoid tissues. (2) Gonadectomy delays thymus involution. (3) Sublethal but sufficient injury of the suprarenal function with intact thyroid prevents thymus involution and causes regeneration even of the involuted thymus. It also causes hypertrophy of other lymphoid tissues. (4) Thyroidectomy prevents thymus regeneration following suprarenalectomy, probably for the same reason that thyroidectomy hastens normal thymic involution.

The first and second of these facts were already known and our studies mainly provide confirmation on a large scale, except that they eliminate the parathyroid factor as influencing the effect of thyroidectomy on the thymus of rabbits.

Recently there has been a tendency to drop the thymus from the group of ductless glands (Sharpey-Schafer (29)) on the grounds that no evidence of an internal secretion exists and that as regards its physiological and pathological reactions it resembles the general lymphoid tissue. While both these statements are true at the present time they indicate our lack of knowledge of this organ rather than a basis for excluding a possible internal secretion. The remarkable con-

stancy with which thymic and general lymphoid hyperplasia follow sublethal injury of the suprarenals and gonads and the constancy with which thyroidectomy prevents it suggest specific chemical interrelationships between the thyroid, thymus, suprarenals, and gonads of both physiological and pathological significance.

These relationships are involved not only in diseases like exophthalmic goiter, acromegaly, Addison's disease, and status lymphaticus, but also in the normal lymphoid hyperplasias of childhood. In all these conditions thymic and lymphoid hyperplasia are prominent features. We have repeatedly pointed out the striking resemblance between the effects of experimental suprarenal injury in rabbits and exophthalmic goiter, especially as regards the thymic and lymphoid hyperplasia and metabolism (30). It is our belief that the constitutional defect underlying the development of exophthalmic goiter, one manifestation of which is lymphoid hyperplasia, is dependent upon partial suppression of a function of the interrenal and sex glands. It is known that this constitutional anomaly may be congenital (inherited?) (31) or acquired, and is either identical with or closely related to status lymphaticus.

Status lymphaticus has been the subject of controversy since Plater (32), in 1614, pointed out that the thymus was often enlarged in individuals dying suddenly without other assignable cause. Kopp (33), in 1838, advanced the view that death was due to the mechanical effects of the enlarged gland. This was vigorously denied in 1858 by Friedleben (34). Paltauf (35), in 1889, first clearly defined the syndrome and advanced the hypothesis that it was a manifestation of a constitutional defect of a chlorotic-lymphatic nature which so lowered the resistance that such individuals were unable to withstand unusual shocks or strains. The work of Hedinger (36) and of Wiesel (37) pointed to the chromaffin tissue as the seat of the primary lesion.

The observations and experiments included in this paper clearly indicate that the essential features of status lymphaticus—abnormal overgrowth of the thymus and lymphoid tissues, lowered resistance, and relative lymphocytosis—have been reproduced experimentally in rabbits. Contrary to the views of Hedinger and Wiesel, we believe that this syndrome develops as a result of insufficient functional activity of the interrenal and sex glands. This insufficiency may be acquired late in life, as shown by its experimental production

in animals and its occurrence in previously normal individuals in association with exophthalmic goiter and Addison's disease, or the functional insufficiency of the interrenal and sex glands may occur during fetal life or infancy. The latter form of insufficiency is responsible for status lymphaticus.

The normal prominence of the lymphoid tissues in infancy and childhood is probably also a manifestation of a functional underdevelopment of the interrenal and gonadal systems and the abnormal hyperplasias of the thymus and lymphoid tissues (status lymphaticus) seen in infants and children are the reactions resulting from more marked insufficiencies of these tissues. It should be pointed out that the prominence of the thymus and lymphoid tissues in infancy is probably related to the normally occurring involution of the interrenal gland in the first few weeks of life (38), which is certainly a powerful stimulus to lymphoid hyperplasia. Status lymphaticus is, therefore, the exaggerated manifestation of a physiological process normal in infants and depends on either a partial loss of or a failure of sufficient development of the interrenal and sex glands.

The thymic and lymphoid hyperplasia occurring in exophthalmic goiter, Addison's disease, status lymphaticus, and even in normal infants has a common cause, and the specific manifestation which differentiates these diseases clinically depends on different degrees of involvement of other specific gland functions. Thus in exophthalmic goiter the disease could be explained as due to a primary interrenal injury with normal or increased functional activity of the chromaffin tissue, both acting to stimulate the thyroid. Addison's disease could be explained as dependent on a combined loss of both chromaffin and interrenal function with possibly, as Wiesel and Hedinger suggest, the chromaffin tissue injury primary. Status lymphaticus is the manifestation of a mild degree of functional underdevelopment of the interrenal, gonadal, and chromaffin tissues.

While we are in the habit of considering exophthalmic goiter as an entity it should be pointed out that the essential symptoms are the recurrence at a later age of physiological processes quite normal for infants. Thus the rate of metabolism and the degree of lymphoid hyperplasia seen in exophthalmic goiter usually do not exceed similar manifestations normally present in infancy.

SUMMARY.

Thyroidectomy hastens, while gonadectomy delays, but does not permanently prevent, involution of the thymus.

Suprarenalectomy alone not only delays involution of the thymus and lymphoid tissue but may cause their regeneration. Thyroidectomy prevents this reaction even after combined suprarenalectomy and gonadectomy.

Suprarenalectomy plus gonadectomy is a more powerful stimulus for thymus and lymphoid regeneration than either of these influences alone.

The combined effect of these two factors results in certain lymphoid and thymus hyperplasia in rabbits which persists until regeneration of accessory interrenal tissue corrects the physiological defect. The syndrome thus experimentally produced resembles status lymphaticus and is believed to depend mainly on a partial loss of certain functions of the interrenal and sex glands rather than of the chromaffin tissue.

The normal and abnormal lymphoid and thymic hyperplasias of infancy and childhood are believed to be manifestations of a functional underdevelopment of the interrenal and sex glands of varying intensity.

The so called lymphatic constitution which underlies or accompanies exophthalmic goiter, Addison's disease, and acromegaly also appears to be dependent on a partial suppression of certain functions of the interrenal and sex glands.

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EXPLANATION OF PLATES.

PLATE 17.

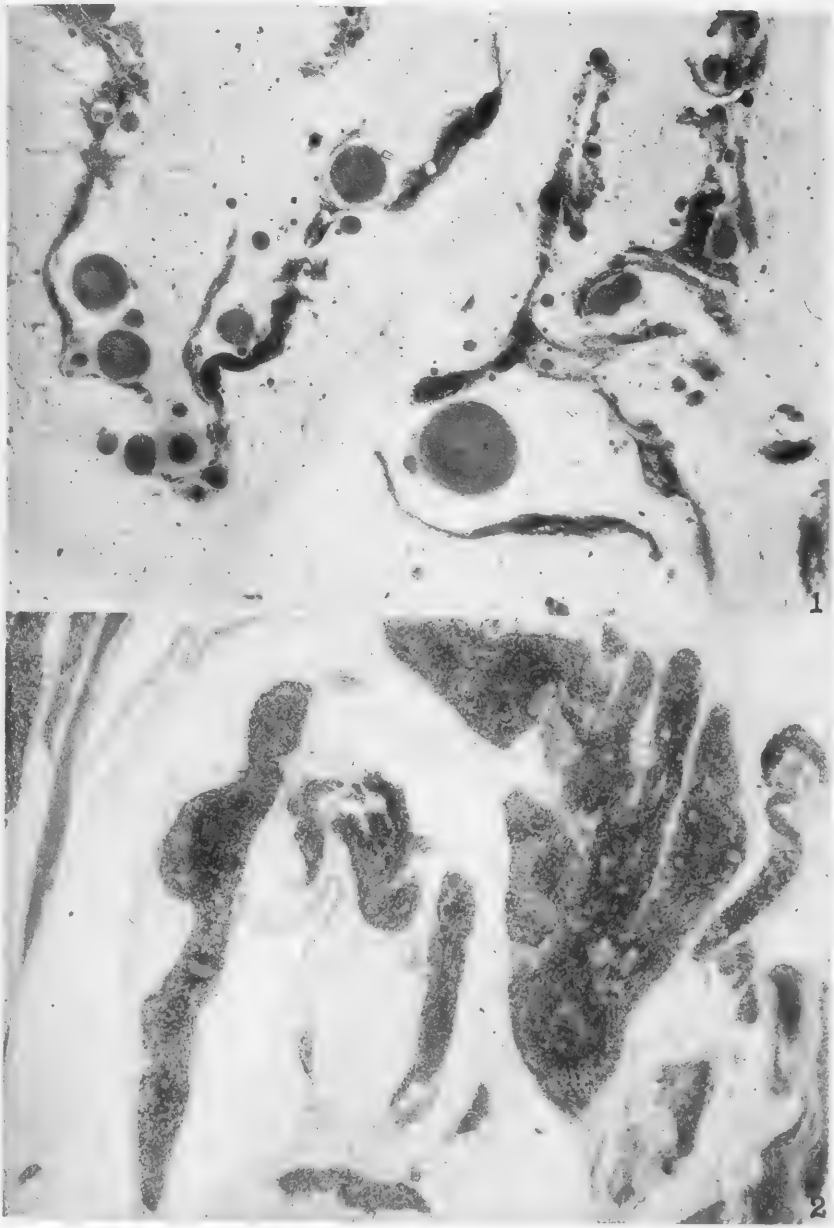
FIG. 1. Rabbit 3–68. Photomicrograph of thymus in complete involution showing the remnants of lobules widely separated by fatty areolar tissue. Indicated in table and text as $+-$. $\times$ 30.

FIG. 2. Rabbit 1–224. Photomicrograph of thymus in advanced involution. Indicated in table and text as $+$. $\times$ 30.

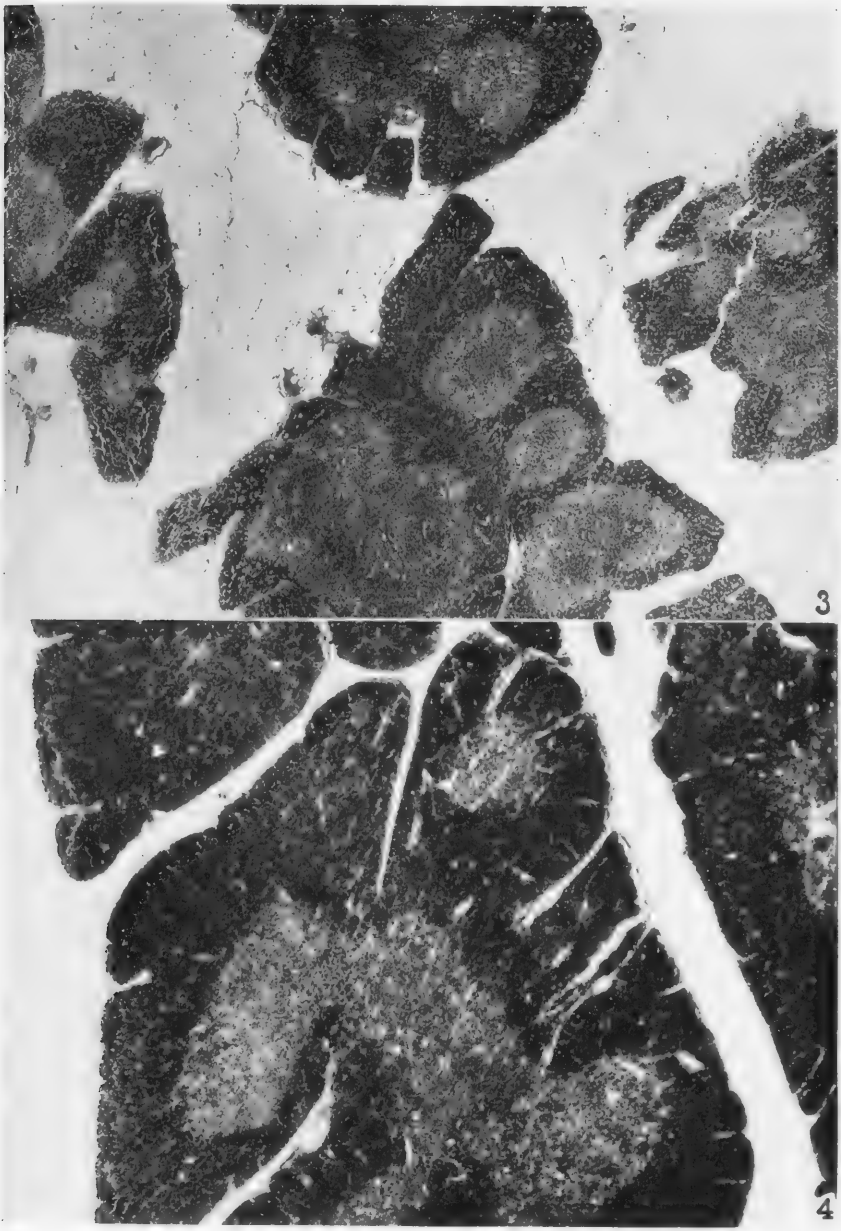
PLATE 18.

FIG. 3. Rabbit 3–270. Photomicrograph of thymus in state of moderate involution (actually regenerating, as indicated by prominent medulla). Indicated in table and text as ++. × 30.

FIG. 4. Rabbit 3–66. Photomicrograph of thymus in state of complete regeneration. Indicated in table and text as +++. × 30.



(Marine, Manley, and Baumann: Thymus gland.)



(Marine, Manley, and Baumann: Thymus gland.)

CLASSIFICATION OF THE HEMOLYTIC STREPTOCOCCI BY THE PRECIPITIN REACTION.

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Following the demonstration by Schottmüller that a useful classification of the streptococci could be made in accordance with their cultural characteristics upon blood agar, a large number of studies have dealt with that family which is defined by the power of its members to produce clear zones of hemolysis surrounding their colonies. That this clinically and pathologically important group should arouse great interest was natural, and that attempts should be made further to analyze its biological properties and if possible to correlate them with the disease pictures produced was obvious.

The earlier studies of this group were concerned largely with morphological, cultural, and fermentative properties. The two former were soon abandoned as means of subclassification, as showing too great variability for practical purposes. Lyall (3), introducing a roughly quantitative method for measuring the degrees of hemolysis produced by various strains, studied their fermentation reactions and observed a measure of uniformity among his cocci; 74.9 per cent fermented salicin alone, while 14.1 per cent more were indifferent to the four carbohydrates employed. Shortly thereafter Holman (4), in promulgating his general classification of the streptococcus family, found that the great majority of his hemolytic strains fell into one group (*Streptococcus pyogenes*), but was able to designate seven variant groups in addition. Obviously such methods were unsatisfactory and temporary, based as they were upon the use of an arbitrarily limited number of substrates. Floyd and Wolbach (2) had previously combined the study of fermentation and agglutination reactions, and, while failing to obtain a more satisfactory grouping of the strains, had observed that there was some degree of correlation possible between the results of the two methods.

Employing the complement fixation reaction, Kinsella and Swift (5) found that their twenty-eight hemolytic strains yielded such marked cross-fixation as to lead to the conclusion that all were closely related or even practically identical, and in

addition showed an orderly relationship to certain members of the non-hemolytic family (6). This was disputed by Howell (7), who was able to demonstrate cross-fixation in only 62 per cent of her tests.

Refinements of technique enabled Dochez, Avery, and Lancefield (9), on the basis of their studies of the agglutination and protection reactions, to demonstrate four major groups of hemolytic streptococci, with a small number of variants among which two further groups were later determined. All the mannitol-fermenting strains fell under one head. Further studies by Dochez and Bliss (10), Bliss (11), and Tunnicliff (12) were directed to the problem of establishing the specificity of those strains associated with scarlet fever.

Davis (1) had observed cross-sensitization in the guinea pig among several strains of the organism under consideration, but confusing cross-reactions appeared between this family and the pneumococci.

The precipitin reaction has received scant attention. Barnes (8) attempted with a fair measure of success to correlate the results of this reaction with those of fermentation, but scrutiny of his results engenders doubts as to the exact nature of the phenomena which he was observing. In view of the striking neglect of this phase of the question, it seemed of interest to study by means of the precipitin reaction a number of strains of hemolytic streptococci, employing as antigens extracts similar to the "residue antigens" described by Zinsser and Parker (13). Similar or identical bacterial products have recently been shown by Avery and Heidelberger (14) to be type-specific within the pneumococcus family. The entire absence of cross-reaction suggests that in these "residues" we possess excellent materials for the group differentiation of bacteria.

Methods.

1. Table I presents the thirty-two strains employed, with reference to their sources and properties.

An effort was made to secure a fairly representative collection of organisms. In every case the cultures were plated out at least twice upon blood agar, and each time single colonies were picked for isolation. Bile solubility tests were made by mixing 0.2 cc. of sterile ox bile with 0.8 cc. of 24 hour plain broth culture, and incubating in the water bath at 37° for 2 hours. For testing the effect upon red cells, 0.5 cc. of a 24 hour culture in plain broth was mixed in a small tube with an equal volume of a 5 per cent suspension of sheep red cells in saline, and the mixture was incubated in the water bath at 37° for 1 hour.

TABLE I.

Sources and Properties of Strains of Hemolytic Streptococci Employed in This Study.

		Bile.	5 per cent sheep cells.	
R80	Rabbit, spontaneous infection.	Insoluble.	Prompt hemolysis.	
S1	Rectum, proctitis.	"	"	"
S2	Throat, follicular tonsillitis.	"	"	"
S3	Chest fluid, pyopneumothorax.	"	"	"
S4	Throat, follicular tonsillitis.	"	"	"
S5	Blood culture, wound infection, septicemia.	"	"	"
S6	Throat, scarlet fever.	"	"	"
S6K	Right kidney (necropsy), scarlet fever.	"	"	"
S7	Uterus (necropsy), puerperal sepsis, peritonitis.	"	"	"
S8	Pus, empyema.	"	"	"
S9	Throat, suspected scarlet fever.	"	"	"
S10	" scarlet fever.	"	"	"
S11	" peritonsillar abscess.	"	"	"
S12	" follicular tonsillitis.	"	"	"
S13	Sputum, bronchopneumonia.	"	"	"
S14	Axillary gland (necropsy), empyema, Hodgkin's disease, septicemia.	"	"	"
S15	Throat, follicular tonsillitis.	"	"	"
S16	Sputum, lobar pneumonia.	"	"	"
S17	Pus, mastoiditis.	"	"	"
S18	Blood culture, puerperal sepsis.	"	"	"
S19	Heart's blood (necropsy), thrombophlebitis, terminal septicemia.	"	"	"
S20	Heart's blood (necropsy), pneumothorax, hemopericardium.	"	"	"
S21	Pus, mastoiditis.	"	"	"
S22	Throat, scarlet fever.	"	"	"
S23	" " "	"	"	"
S24	" " "	"	"	"
S60	" measles.	"	"	"
S107	Sputum, "	"	"	"
S139	Throat, bronchopneumonia.	"	"	"
S177	" follicular tonsillitis.	"	"	"
S212	" " "	"	"	"
S222	" " "	"	"	"

2. Bacterial extracts for use as antigens in the precipitin reaction were prepared as follows:

The organisms from a 24 hour culture in 1 per cent glucose beef infusion broth were sedimented by centrifugation, washed once with physiological saline, and recentrifuged. The packed organisms were desiccated overnight *in vacuo* over phosphoric anhydride; the dry residue was finely ground in a small mortar, and was weighed to the nearest milligram. It was then extracted with 0.8 per cent antiformin, 0.5 cc. per mg. of dried organisms, in the water bath at 56°, for 10 minutes, being shaken vigorously two or three times during the extraction. The suspension at the end of the designated time was treated with a 5 per cent solution of sodium thiosulfate, 3 drops being used per cc. of diluted antiformin; it was then rapidly cooled to room temperature under the tap. $\frac{N}{5}$ sulfuric acid was added slowly and with gentle agitation until a heavy flocculent precipitate appeared. At this point the mixture was tested with litmus paper, and, if necessary, further acid was added drop by drop until a *faint* acid reaction appeared. The liquid was then centrifuged for a few minutes at moderate speed, and the clear supernatant solution was removed by decantation. This solution was brought to the neutral point with a drop or two of $\frac{N}{5}$ sodium hydroxide, and saline containing 0.5 per cent phenol was added until the final volume was such that 1 cc. represented 1 mg. of the original desiccated organism. This method was strictly adhered to, in the hope that the final solutions would be comparable in a roughly quantitative sense.

As different lots of antiformin have been found to vary appreciably in their available chlorine content, and as the preparation of a satisfactory extract is more dependent upon this factor than upon that of alkalinity, it was found advisable to titrate each new lot in order to obtain the best results. 10 cc. of the diluted antiformin were measured into a small flask, and to this were added 0.25 gm. of solid KI and 1 cc. of strong acetic acid. The liberated iodine was titrated with $\frac{N}{10}$ sodium thiosulfate, with weak starch solution as indicator. If the available chlorine was found to be approximately equivalent to 1.7 cc. of the standard thiosulfate, the dilution of antiformin was considered satisfactory for use.[1]

3. Rabbits were used for the preparation of antisera. Immunization was effected by the intravenous injection of whole 18 hour cultures in plain meat infusion broth of pH 7.6, sterilized by heating in the water bath at 56° for 1 hour. After considerable experimentation, the following method was adopted as yielding the most satisfactory results.

[1] The author is greatly indebted to Dr. A. P. Briggs for suggesting this method of titration.

On 4 successive days the animals were given injections of 1, 2, 5, and 10 cc. respectively. After a lapse of 5 days followed a further course of four doses, the first two of which were of 10 and 20 cc. respectively. The third and fourth doses consisted of the organisms sedimented by centrifugation from 50 cc. of plain broth culture and resuspended in 10 cc. of the supernatant liquid. After the further lapse of 1 week, the animals received three injections, each consisting of the organisms sedimented from 100 cc. of broth culture and resuspended in 10 cc. of the supernatant liquid. Trial bleedings were made 1 week following the last dose. If the precipitin content of the serum was not satisfactory, the last series of three injections was repeated once or twice, at weekly intervals, and the animals were bled 1 week following the last injection. In every case the rabbits responded by the production of precipitating antibodies, although there was considerable individual variation in the potency of the sera obtained. In all, nine satisfactory sera were prepared.

4. Precipitation tests were performed as follows:

0.2 cc. of the serum at hand was placed into each of a number of small tubes, and to each tube was added 0.2 cc. of antigen prepared as above outlined. The contents were mixed by gentle shaking. Positive results were denoted by the immediate clouding of the mixture. With the most potent sera, rapid flocculation appeared, and within an hour at room temperature the lower third of the tube was occuped by a heavy, coarse precipitate. The tubes were allowed to stand at room temperature for several hours, and were then placed in the ice box overnight before the final reading.

RESULTS.

These are presented in summary in Table II.

In every case there was made a series of control tests, with 0.2 cc. of normal rabbit serum in place of the immune serum. Furthermore, each immune serum was tested with antigens prepared as above from each of sixteen strains of non-hemolytic streptococci. In no instance was there any indication of cross-precipitation or of any non-specific flocculation.

Of the thirty-two strains of true hemolytic streptococci employed in this study, thirty-one gave immediate, constant, and practically uniform precipitation with each of the immune sera at hand. After standing several hours in the ice box, the precipitate was found to consist either of definite coarse flakes which could easily be observed by gentle agitation of the tube, or else of a densely matted mass which could not be broken up even by violent shaking. One strain con-

TABLE II.

Hemolytic Strains.

	R80	S1	S2	S3	S4	S5	S6	S6K	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	S60	S107	S139	S177	S212	S222
Serum R80....	3	3	3	3	3	3	3	3	3	0	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
" S1.....	3	3	3	3	3	3	3	3	2	0	2	3	3	3	3	3	3	3	3	3	2	2	2	3	3	3	3	3	3	2	3	3
" S2.....	4	4	4	4	4	4	4	4	4	0	4	4	4	4	4	4	4	4	4	4	4	4	3	3	3	4	4	4	4	4	4	4
" S3.....	4	4	4	4	4	4	4	4	4	0	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	3	4	4	4	4	4	4
" S7.....	3	3	2	3	3	3	3	3	2	0	3	3	3	3	3	3	3	3	3	3	3	2	2	3	3	2	3	3	3	3	3	2
" S16.....	3	3	3	3	3	3	3	3	3	0	3	3	3	3	3	3	3	3	3	3	3	3	3	2	2	2	3	3	3	3	3	3
" S22.....	3	3	3	3	3	3	3	3	3	0	3	3	3	3	3	3	3	3	3	3	2	3	3	3	3	3	3	3	3	3	3	3
" S23.....	3	3	3	3	3	3	3	3	3	0	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
" S139....	3	3	2	2	2	2	2	2	2	0	2	3	3	3	2	2	3	2	2	2	2	2	2	2	2	3	2	2	2	2	2	2
Normal rabbit serum.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Non-Hemolytic Strains.

	A4	A49	A135	A179	B1	2B	B3	B4	C1	2C	C3	2D	38D	MB	R	W72
Serum R80.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S1.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S2.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S3.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S7.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S16.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S22.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S23.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S139.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Normal rabbit serum.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

4 indicates heavy, matted precipitate, not fragmented upon agitation; 3, the same but less in amount; 2, flocculent precipitate, fragmented upon agitation; 0, no precipitate.

stantly failed to give positive results even with the strongest sera. Further study of this organism has shown it to be very weak antigenically, and only with difficulty could antisera to it be produced. Such sera yielded weak precipitation and complement fixation reactions with antigens made from the homologous organism, but were entirely devoid of any cross-reaction whatsoever.

DISCUSSION.

This study indicates that on the whole the hemolytic streptococci form one homogeneous group, so far as may be concluded from their property of yielding a common precipitating substance. The existence of variant strains lacking this property is indicated, but undoubtedly they are greatly in the minority. This uniformity is strikingly in contrast with the properties of the non-hemolytic family, which, as will be shown elsewhere, are quite heterogeneous when studied by the same method. The entire absence of cross-reaction between the two groups stands in sharp contrast to the results which have been obtained by means of the complement fixation reaction, in which were used as antigens complete solutions of streptococci in stronger antiformin (6). The solutions prepared as above described are practically free from protein, and it is without doubt this feature which is accountable for their type specificity. Avery and Heidelberger (14) have shown that the protein of the pneumococcus is specific for species but not for type, and observations indicating that the same statement holds true for the streptococci will be published in the near future.

No attempt has been made to study the present series by agglutination, protection, or fermentation reactions. These have been so thoroughly investigated by others that it has seemed unnecessary to to resort to them. There is no reason to question the existence of subgroups within the family of hemolytic streptococci; indeed, a parallel case is afforded by the existence of subtypes of Pneumococcus Type II, all yielding good precipitation reactions with the standard type serum.

SUMMARY AND CONCLUSIONS.

A method is described for the preparation from hemolytic streptococci of extracts containing a soluble precipitating substance.

By the use of such extracts, it is shown that nearly all members of this family yield a common precipitable substance.

So far, therefore, as concerns the precipitin reaction, the hemolytic streptococci form a practically homogeneous group.

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STUDIES IN AGGLUTINATION.

II. The Relationship of Reduction of Electrical Charge to Specific Bacterial Agglutination.

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INTRODUCTION.

Environment plays a most important part in the immunological reaction of agglutination. Studies are being undertaken to determine the relationship of this factor to the optimum effectiveness of the test. The general principles underlying this relationship, and the mode of attacking the problem have been set forth in a previous paper (1).

With a view to a better understanding of the mechanism of the reaction, it was deemed advisable to study the nature of the changes in electrical charge on the bacteria, which accompany the phenomenon of specific bacterial agglutination.

This aspect of agglutination has been studied by many investigators. Bechhold (2) observed that sensitized bacteria lost their power to migrate in an electrical field, that is lost their charge; his experiments were conducted by the macroscopic cataphoretic method, which is not wholly satisfactory, and the importance of salt effects was given insufficient consideration. Neisser and Friedemann (3) made similar assertions. Recently, Northrop and De Kruif (4–7) in their very careful study of the stability of bacteria in suspension have investigated these effects in detail and have contributed much to an understanding of both specific and non-specific agglutination. Before considering our experimental results, it would be well to review briefly the conception of bacterial agglutination which they have formulated.

According to these writers, agglutination may be considered in terms of two antagonistic forces, a *repelling* force which keeps the bacteria apart, due to like

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charge on the bacteria, and a *cohesive* or sticking force, which is probably a function of surface tension. In any bacterial suspension these forces may be thought of as opposed to each other; greater relative repelling force making for stability, and greater relative cohesive force leading to flocculation. They have shown, in the case of both unsensitized and sensitized bacteria, that agglutination takes place as soon as the potential is reduced (by electrolytes) to a critical zone lying between about −15 and +15 millivolts, provided the cohesive force is unaffected; that is, the cohesive force then becomes relatively greater than the repelling force. They have shown, in the case of unsensitized bacteria, that salt in high concentration (over 0.01 N) reduces the cohesive force and, at the same time, narrows the critical potential agglutination zone; but that, in the case of sensitized bacteria, such reduction of cohesive force and of potential agglutination zone does not take place. And they have found further that all electrolytes tested in low concentration (less than 0.01 to 0.1 N) affect primarily the potential, while, in concentrations greater than this, the effect is principally on the cohesive force.

As a result of preliminary observations, we believed that specific bacterial agglutination might occur outside of the potential agglutination zone just referred to. In the experiments reported below this possibility is demonstrated, and, in addition, evidence is produced to indicate that specific agglutination may occur independently of charge reduction, and paradoxically, that immune agglutinating sera possess specific charge-reducing effects.

Experimental Methods.

Measurement of Potential.—The potential was determined from the rate of migration, with the Northrop micro cataphoresis cell (4). The potential gradient was 4.23 volts per cm. Calculation of P.D. was made in accordance with the formula of Northrop and Cullen (8). Since some doubt exists regarding the correctness of the Helmholtz-Lamb equation, from which this formula is derived, for the calculation of potential from migration rate,[1] the results are given both in terms of millivolts and of observed migration velocity, in microns per second. The technique followed was that recommended by Northrop with one exception. Theoretically the cell is symmetrical, and when small particles are being cataphoresed, readings may be made in either the three upper or lower sixths. Owing to unequal coating by serum and bacteria, and particularly by bacterial clumps, slight differences between the two halves were sometimes noted;[2] accordingly our readings were made at all six levels.

[1] Full discussion of the reasons for this doubt is given by Northrop and Cullen (8), Northrop and De Kruif (5), and Winslow, Falk, and Caulfield (10).

[2] Eggerth (11) similarly notes the need for this precaution.

Buffers Used.—Glycocoll, sodium phosphate, sodium acetate (G.P.A.) mixture (5); citric acid-sodium phosphate mixture (9).

pH Determinations.—These were done colorimetrically.

Agglutination Methods.—The macroscopic method was used. The degree of agglutination was indicated as follows: C., complete agglutination, supernatant fluid clear; 3, marked granular appearance with beginning flocculation; 2, the same, without flocculation; 1, slight granular appearance to the naked eye; ± doubtful agglutination, slightly granular with hand lens. + and − are used to indicate intermediates between these. In the text-figures, C. is indicated by a solid line, 3 to 1 by an interrupted line, and no agglutination by a dotted line.

Bacteria Used.—Type I, Type II, and Type III pneumococcus; *B. paratyphosus* A and B; Diplococcus NY5, a streptococcus variant.

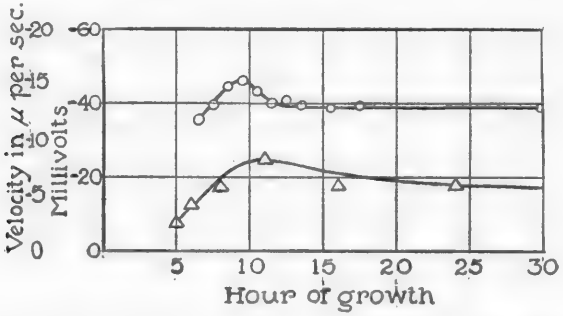

FIG. 1. Relationship of age of culture to charge on bacteria. Upper curve, Type I pneumococcus; lower, *B. paratyphosus* B.

Media.—Stock beef broth, buffered with sodium phosphate, pH = 7.6.

Age of Cultures.—Early in the experiments, it was found that the charge on the bacteria varied markedly from time to time, and that, occasionally, there were slower and faster rates of migration at the same level in the cell. The age of the culture was suspected of being the factor and investigation showed this to be the case. The charge of bacteria was determined at different hours of growth, and was found to be low in young cultures, to reach a peak in 10 to 12 hours, and to drop to, and maintain, a fairly constant level thereafter for 48 hours. Once or twice mixed migration rates were observed in old cultures (24 to 48 hours). Accordingly 16 to 18 hour cultures were used in all experiments.

Fig. 1 shows the relationship of growth to charge in the cases of Type I pneumococcus and *B. paratyphosus* B. The bacteria were washed twice in distilled water and were then resuspended in distilled water before the charge was determined.

Washing of Bacteria.—All bacteria used in the experiments were washed two to four times in distilled water.

EXPERIMENTAL RESULTS.

One of the objects of the investigation was to determine if specific agglutination could take place outside of the potential agglutination zone referred to above. Therefore, a high initial charge on the bacteria was desired. Thoroughly washed bacteria, suspended in distilled water, carry a relatively high negative charge (20 to 50 millivolts). Electrolytes, however, are essential for agglutination with immune serum (12); but, when ordinary salts, in sufficient concentration for the occurrence of flocculation, are used, they lower the charge on the bacteria to -15 millivolts or less (*i.e.* into the zone of Northrop and De Kruif). However, if bacteria are suspended in buffer mixtures of pH 6.0 to 8.0, they have a high charge, although the electrolytes present be relatively concentrated. Accordingly, buffer mixtures of pH 7.0 and 7.6 were used in the first group of experiments. This choice was advantageous as the pH of the serum falls in this zone. The fact that the buffer mixtures contained phosphate was fortunate, as will be brought out below. The total salt concentration was kept fairly low, M/100 to M/200, in order to minimize the depressing effect upon potential and cohesive force.

In the first group of experiments there are shown the effects of normal and immune sera upon the potential and the agglutination of homologous and heterologous organisms.

Fig. 2 shows the effect of normal serum and Type I pneumococcus serum[3] upon Type I pneumococcus. It will be seen that both sera depress the charge on the bacteria, but that the normal serum effect is only slight, while the specific serum reduces the charge in a striking way. Agglutination by the immune serum was complete in dilutions 1:100, 1:200, and 1:400, and was incomplete in 1:50 (prozone) and 1:800. The prozone effect was noted in other experiments and is very interesting in view of the fact that the charge on the bacteria is in the potential agglutination zone of Northrop and De Kruif. It will be noted further that agglutination took place in dilutions 1:100 to 1:400 although the charge on the bacteria was

[3] Diagnostic serum obtained from The Rockefeller Institute for Medical Research.

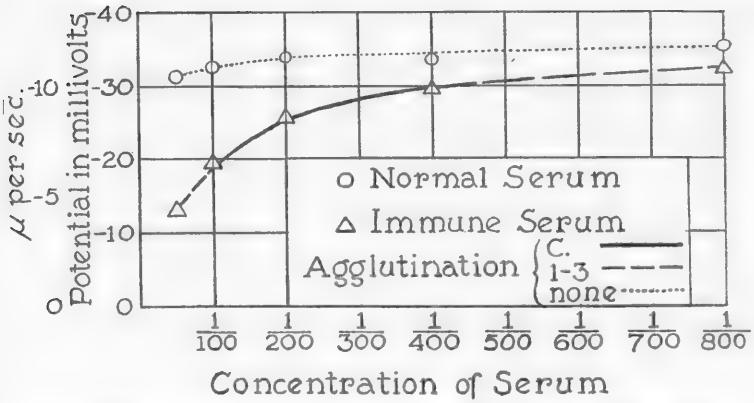

FIG. 2. Effect of normal serum and Type I pneumococcus immune serum on potential and agglutination of Type I pneumococcus.

TABLE I.

The Time Factor in the Lowering of Charge on Bacteria by Immune Serum.

Type I pneumococcus serum and Type I pneumococcus were used. The mixtures of the bacteria were made in G. P. A. buffer, pH 7.6, total salt concentration M/200.

Concentration of immune serum.	Potential. Time of determination.		
	Immediate.	At 2 hrs.	At 16 hrs.
	mv.	mv.	mv.
1:20	−11.1	−11.3	−10.5
1:40	−12.8	−12.9	−12.3
1:80	−14.4	−15.1	−14.7
1:160	−19.5	−18.9	−18.4
1:320	−28.4	−28.5	−29.1
None.	−29.1	—	—

well above −15 millivolts. The time factor in the lowering of charge was studied, and it will be seen from Table I that the reduction is immediate, and that the levels reached, in the different serum dilutions, were maintained for the duration of the experiment (16 hours). All immediate determinations were done in the space of 2 to 4 minutes

after the mixtures were made; in one case, readings were started within 16 seconds. No difficulty was experienced in determining the velocity of clumped bacteria (in late readings), provided that the suspensions were well shaken and that the lowest levels were taken first; that is, before settling in the cell had taken place.

Fig. 3 shows the effect of specific immune serum[4] upon *Bacillus paratyphosus* A. The findings are essentially the same as noted above. Two points are worthy of note: (1) the charge-reducing effect of normal serum disappeared at about 1:1,000 dilution, but was still present in the immune serum above a dilution of 1:6,400; and (2) almost complete agglutination (C.−) occurred at a dilution of 1:12,800, although the charge on the bacteria was as high as that of the organisms untreated with serum. In other words, it would seem that we have here specific agglutination occurring independently of reduction of charge. This experiment was repeated with dialyzed sera.[5] The results were essentially the same (Fig. 3), although the general level of charge reduction was higher than in the experiment with electrolyte present. As was to be expected, agglutination with the immune serum, even in the 1:50 dilution, did not take place, in view of the absence of electrolyte.[6] A similar experiment with NaCl, M/14 (0.45 per cent), showed similar differences between the immune and normal serum effects, although here all charges were low, −14 millivolts or less, as a result of the high salt concentration.

The specificity of the reaction was now tested. Fig. 4 shows the effect of Type I pneumococcus immune serum, and of normal serum, upon Type I, Type II, and Type III pneumococcus. The striking difference between the specific and the normal serum effects upon the Type I organism is shown in the upper curves (*A*). The lower curves, (*B*, *C*) show that the Type I serum acts essentially as normal serum in the case of Type II and Type III pneumococcus. Type I serum

[4] Immune rabbit serum, agglutination titer 1:25,000.

[5] The sera were dialyzed in collodion sacs 3 days against running tap water and 2 days against distilled water.

[6] Northrop and De Kruif (6) studying dialyzed serum observed similar absence of agglutination. It is interesting to note that their curves in this experiment show differences between the charge-reducing effect of normal and immune sera comparable to those obtained by us.

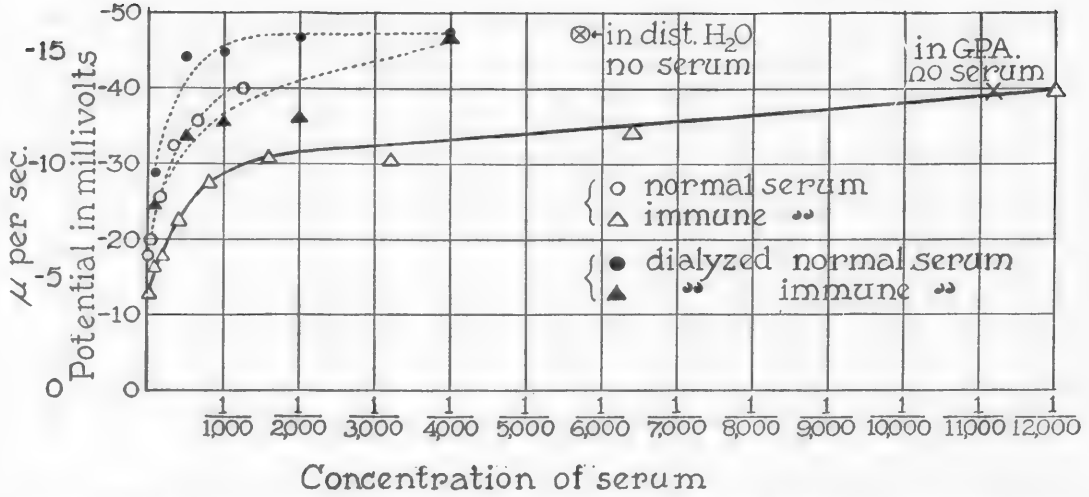

FIG. 3. Effect of normal serum and *B. paratyphosus* A serum on potential and agglutination of *B. paratyphosus* A. The results of two experiments are shown, one with undialyzed serum in G.P.A. buffer pH = 7.0 (M/200) and one in distilled water with dialyzed serum.

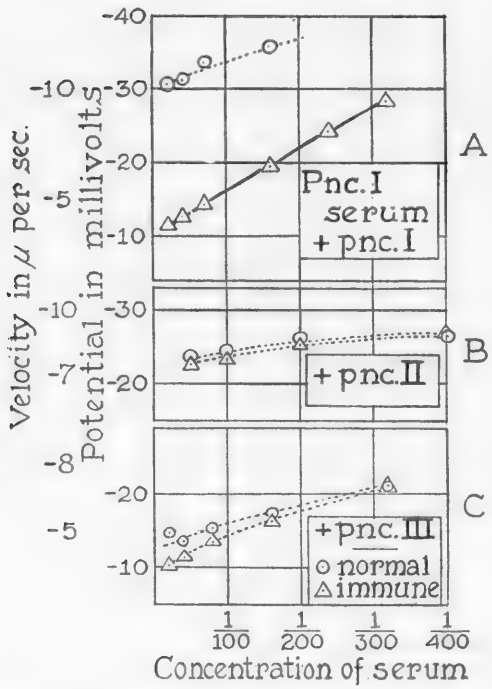

FIG. 4. Effect of normal serum and Type I pneumococcus immune serum on agglutination and potential of Type I, Type II, and Type III pneumococcus.

TABLE II.

Charge-Reducing Effect of Type I Pneumococcus Serum and of Diplococcus NY5 Serum on Diplococcus NY5, and Pneumococcus I.

Experiment with G. P. A. buffer, pH 7.6, M/200.

Serum.	Bacteria.	Potential.	Agglutination.
		mv.	
Type I pneumococcus, 1:40.	Pneumococcus I.	−13.4	1+*
" " " 1:40.	Diplococcus NY5.	−28.9	−
Diplococcus NY5, 1:40.	" "	−7.0	C.
" " 1:40.	Pneumococcus I.	36.2	−

* This is in the prozone; complete agglutination occurred in higher dilutions.

was also tested against Diplococcus NY5 (Table II), and it will be seen that it acts like normal serum, while the specific diplococcus serum reduces the charge markedly. These results indicate that the charge-reducing effects observed are highly specific.

The next experiment shows that this charge-reducing effect is probably a property of immune serum, provided only, that such serum possesses agglutinating power. Guinea pig serum which was highly protective for guinea pigs against Type I pneumococcus (0.5 cc. protected against 1,000,000 lethal doses), but which did not agglutinate this organism, was tested with Type I pneumococcus. Table III shows that this serum lowers the charge on the bacteria only to about

TABLE III.

Charge-Reducing Effect of Non-Agglutinating, Protective Serum.

Type I pneumococcus immune guinea pig serum used with Type I pneumococcus. 0.5 cc. serum protected a guinea pig against 1,000,000 lethal doses but had no agglutinating power. Experiment with G. P. A. buffer, pH 7.6, M/200.

Serum.	P.D.	Agglutination.
	mv.	
Normal, guinea pig, 1:20.	−27.8	−
Immune, " " 1:20.	−26.0	±
None.	−33.1	−
Immune, horse (agglutinating).	−11.1	1+*

* This is in the prozone; complete agglutination occurred at higher dilutions.

the same degree as normal guinea pig serum. The marked charge-reducing effect of specific agglutinating serum, in the same dilution, is shown for comparison.

The close relationship between the agglutinin of immune serum and its charge-reducing effect is clearly demonstrated in Fig. 5. The charge-reducing effects of specific agglutinating sera, Type I pneumococcus and *Bacillus paratyphosus* A, and of normal serum, were determined first. The immune sera were then adsorbed with their homologous organisms in the usual way. These adsorbed sera, now deficient in agglutinating power, were again tested, and it was found that their previous marked reducing effect had disappeared, and that they behaved in a fashion similar to that of normal serum. A control experiment with *Bacillus paratyphosus* A serum, in which adsorp-

tion was done with the heterologous organism, *Bacillus paratyphosus* B, failed to show a similar loss of the charge-reducing effect.

The fact that immune agglutinating sera possess this specific charge-reducing power suggests that the effect may have practical diagnostic value. This would be true especially in the case of autoagglutinating and so called inagglutinable bacteria. We plan to study this aspect of the problem in the near future.

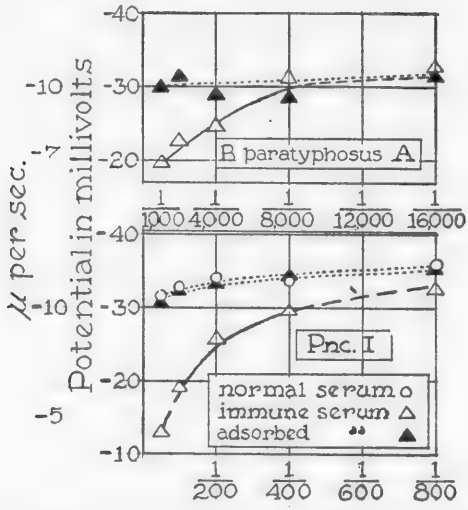

FIG. 5. Effect of adsorption of agglutinin on the charge-reducing effect of Type I pneumococcus and *B. paratyphosus* A immune sera. In the case of the former, the normal serum effect is shown as a control; in the latter the normal serum effect disappears at the dilutions tested.

From the foregoing experiments, it is clear that specific agglutination may take place when the charge on the bacteria is well over a value of −15 millivolts. This is not in keeping with the results, detailed above, of Northrop and De Kruif. At the suggestion of Dr. Northrop, we repeated some of his experiments (6) with the hope of finding that the potential agglutination zone had been widened by the immune serum.[7] The experiments were carried out with a

[7] This possibility was suspected from studies Dr. Northrop then had under way, in which it was observed that specific, agglutinating, red blood cell serum markedly widens the potential agglutination zone of red blood cells (13).

constant serum dilution (*Bacillus paratyphosus* A serum, 1:2,000), and the electrolyte concentration was varied; NaCl, $ZnSO_4$, and $CeCl_3$ were used. Fig. 7 shows the $ZnSO_4$ curve. Our results showed that the potential zone was not widened, complete agglutination occurring *only* when the potential fell to a value below −15 millivolts. These findings confirm the results of Northrop and De Kruif. In an effort to explain the opposing results, buffer mixtures (G.P.A.), of varying pH, were now substituted in the experiment (Fig. 6), and, with immune serum, agglutination *was obtained again at values*

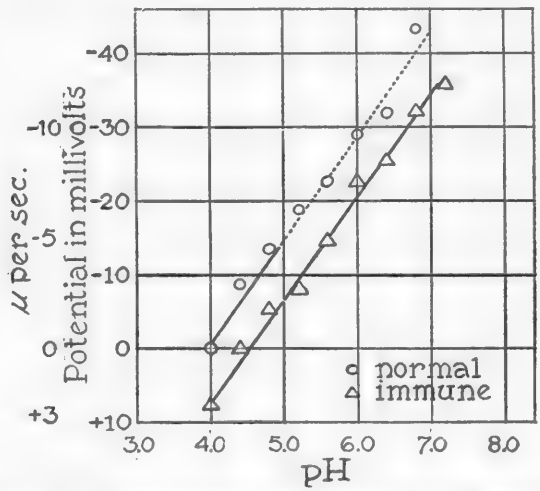

FIG. 6. Effect of pH on agglutination and potential of *B. paratyphosus* A, treated with 1:2,000 normal and immune serum. Concentration of buffer mixtures was M/200.

over −15 millivolts, −36 millivolts at the upper limit of the experiment. The normal serum curve shows agglutination taking place as soon as the potential falls below −15 millivolts (acid agglutination zone). Results, similar to these, were obtained with the sodium phosphate-citric acid buffer.

Sodium phosphate was the only salt common to the two buffers. Accordingly, the experiment was repeated with this electrolyte alone. Fig. 7 shows the results. It will be seen that this salt behaves quite differently from NaCl, $ZnSO_4$, and $CeCl_3$ (in our experiments) and

from the salts used by Northrop and De Kruif (6). In the case of all these salts, agglutination takes place only when the potential falls below −15 millivolts; while, with Na_2HPO_4, agglutination occurs as high above this critical value as −34.6 millivolts. This charge was approximately that on the bacteria in distilled water, without serum, and is an additional point in favor of the assumption that specific agglutination may take place independently of charge reduction. The effect of $ZnSO_4$ is shown in Fig. 7 for comparison.

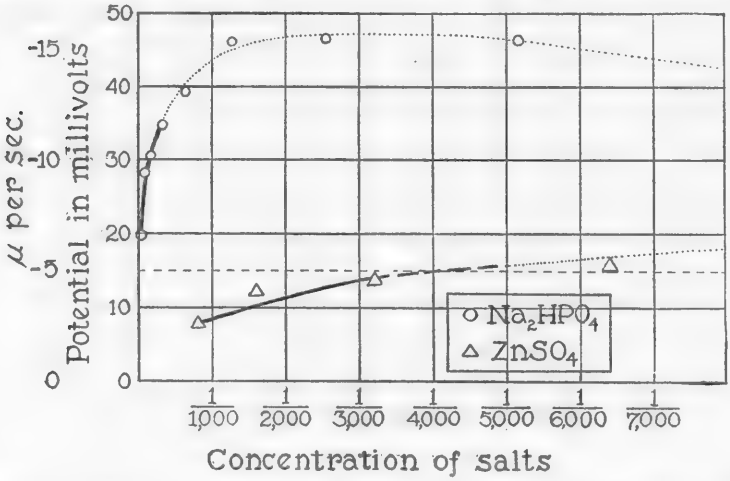

FIG. 7. Effect of concentration of Na_2HPO_4 and of $ZnSO_4$ on potential and agglutination of *B. paratyphosus* A, treated with specific serum, 1:2,000. Charge on bacteria in distilled water in the case of the former was 34.0 millivolts.

The reason for the different behavior of these salts may lie in the fact that the high pH of the phosphate solutions (7.4 to 7.9 in dilutions in which agglutination occurred) acts to maintain a high charge, while the electrolyte is concentrated enough to permit agglutination. An experiment similar to the preceding, with $NaHCO_3$, in which agglutination was complete up to a charge of −22.8 millivolts, bears out this possibility. We are planning further studies in this aspect of the problem.

SUMMARY AND CONCLUSIONS.

1. The effect of immune serum upon the electrical charge on bacteria has been studied.

2. Immune *agglutinating* serum possesses a specific charge-reducing effect which is quantitatively related to the agglutination titer of the serum. This effect is lost when the serum loses its agglutinating power, that is after adsorption of agglutinin by homologous bacteria; adsorption by heterologous organisms does not affect this property.

3. A highly protective, non-agglutinating serum did not show this specific charge-reducing effect.

4. It is suggested that the reaction may have practical diagnostic application.

5. The findings of Northrop and De Kruif, that unsensitized and sensitized bacteria agglutinate only when the charge on the bacteria has been reduced to a critical potential zone lying between +15 and −15 millivolts, have been confirmed in the case of all electrolytes tested except Na_2HPO_4.

6. When Na_2HPO_4 or phosphate buffer solutions are used as electrolytes, specific bacterial agglutination occurs at negative charges well above −15 millivolts, and with serum in high dilution, specific agglutination takes place without any observable reduction of charge.

The writer wishes to express his indebtedness to Dr. J. H. Northrop, of The Rockefeller Institute for Medical Research, for many valuable suggestions in the course of the investigation.

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STUDIES ON PNEUMOCOCCUS GROWTH INHIBITION.

III. The Influence of Specific Antipneumococcus Serum on the Growth-Inhibitory and Bactericidal Action of Normal Serum-Leucocyte Mixtures.

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In the preceding paper[1] a method was described for demonstrating the growth-inhibitory and pneumococcidal effects of mixtures of serum and washed leucocytes from animals naturally resistant to pneumococcus infection. Serum and leucocytes of susceptible animals were found to lack these properties. The present work is a continuation of the study and deals with the action of pneumococcus immune serum in serum-leucocyte mixtures of a susceptible animal—the rabbit. The different views concerning the mechanism by which antipneumococcus serum produces its protective effects and the literature on previous investigation of the pneumococcidal activity of the blood of artificially immunized animals were presented in the paper above mentioned. Suffice it to say here that there is no general agreement among workers in this field as to whether the increased resistance of animals, actively or passively immunized against pneumococcus, is due to the acquisition of pneumococcus-destroying power by the blood or is to be attributed to some other and possibly unknown protective mechanism.

Technique.

The technique employed in the following experiments was the same as that previously used. The organism was Pneumococcus Type I of a strain which killed rabbits in quantities of 0.000001 cc. of a standard suspension. Rabbit leucocytes, obtained from an aleuronat pleural exudate, were used throughout. The normal rabbit serum was secured within 24 hours of use either from normal

[1] Robertson, O. H., and Sia, R. H. P., *J. Exp. Med.*, 1924, xxxix, 219.

rabbits weighing 1,500 to 2,000 gm., or from the one supplying the leucocytes. Except when it is otherwise stated, the immune serum used in the following experiments was the therapeutic antipneumococcus serum Type I supplied by the New York State Department of Health. Two lots of serum were employed —one dated February, 1919, and the other November, 1922.[2] The latter serum was used in all the experiments except Nos. 1 and 8, in which the 1919 serum was employed. Both these immune sera were tested for their mouse protective properties at the beginning of the present study, and were found to be highly potent—0.2 cc. of serum protected Chinese field mice, weighing 20 gm., against 0.2 to 0.4 cc. of the standard suspension of pneumococci which killed in quantities of 0.0000001 cc. The immune serum dilutions were made Locke's or gelatin-Locke's solution. Save when otherwise indicated, the immune serum was not inactivated by heating.

EXPERIMENTAL.

Bactericidal Action of a Normal Rabbit Serum-Leucocyte Mixture Containing Immune Serum.

Experiment 1.—(Table I.) Two strains of Type I pneumococcus of different virulence for rabbits were tested. Strain A is the original Neufeld obtained from the Hospital of The Rockefeller Institute where it had been kept in blood broth for a number of years. Its killing dose for rabbits was found to be 0.01 to 0.001 cc. of an abundant actively growing culture. Strain B is the organism used regularly throughout the following experiments, one of high virulence for rabbits. The immune serum was inactivated at 56°C. for ½ hour. Agitation was maintained at a rate of 35 to 40 revolutions and 8 to 10 oscillations per minute continuously for a period of 72 hours, at the end of which time the contents of the tubes were examined microscopically and transplanted into dextrose blood broth.

The addition of a very small quantity of immune serum—0.0001 cc.—to a rabbit serum-leucocyte mixture was found to confer on this medium marked growth-inhibitory powers. Table I shows that pneumococci in quantities less than 0.001 cc. of the standard suspension failed to show any growth at the end of 72 hours. Furthermore, cultures of the contents of such tubes as failed to show organisms in the stained film at the end of this time proved negative. On the other hand, control tubes containing rabbit leucocytes and serum without immune serum failed to show inhibition of the growth of even 0.0000001 cc. of the same suspension of pneumococci. The two strains of

[2] These experiments were carried out in 1923. The immune sera had been kept in the ice box except during the period of transit to China.

pneumococci, differing considerably in virulence, reacted alike in these regards.

In order to exclude the possibility that lack of growth in the dextrose blood broth at the end of 72 hours might be due merely to involutional

TABLE I.

Bactericidal Action of Rabbit Serum-Leucocyte Mixture Containing Immune Serum.

Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + immune serum dilution 0.1 cc. + pneumococcus suspension 0.1 cc.

	Amount of standard suspension.	Amount of immune serum.	Color change. At hrs. 24	48	72	At 72 hrs. Pneumococci in stained film.	Growth in culture.
	cc.	cc.					
(*a*)	0.01	0.0001	++++			+	
Moderately	0.001	"	0	++++		+	
virulent	0.0001	"	0	0	0	0	0
organism.	0.00001	"	0	+*	+*	0	0
	0.000001	"	0	0	0	0	0
(*b*)	0.001	"	0	++	++++	+	
Highly	0.0001	"	0	0	0	0	0
virulent	0.00001	"	0	0	0	0	0
organism.	0.000001	"	0	0	0	0	0
	0.0000001	"	0	0	0	0	0
	Control without immune serum.						
	0.0000001		++++			+	

Color changes indicating formation of methemoglobin are indicated as follows:

±, first appreciable change—a little darker red.

+, well marked change—definitely darker.

++, moderately deep red.

+++, very deep purplish red.

++++, black.

* Hemolysis.

changes incident to the long inhibition of growth in the serum-leucocyte mixture and hence could not be taken to indicate a direct pneumococcidal action, the contents of the tubes in Experiment 2 were cultured at varying intervals of time from 12 to 72 hours.

TABLE II.

Tests for Bactericidal Action at Varying Intervals of Time.

Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + immune serum dilution 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Amount of immune serum.	Culture.	Pneumococci in stained film at time of culture.	Growth in culture.	Culture media.
cc.	*cc.*	*hrs.*			
0.01	0.001	12	−*	+	Dextrose blood broth.
0.001	"		−	+	
0.0001	"		−	0	
0.00001	"		−	0	
0.000001	"		−	0	
0.01	"	24	−	+	" " "
0.001	"		0	+	
0.0001	"		0	0	
0.00001	"		0	0	
0.01	"	48 and 72	+		" " "
0.001	"		0	0	
0.0001	"		0	0	
0.00001	"		0	0	
0.01	"	24	+	+	" " agar
0.001	"		0	0	plate.
0.0001	"		0	0	
0.00001	"		0	0	
0.01	"	48 and 72	+		Dextrose blood agar
0.001	"		0	0	plate.
0.0001	"		0	0	
0.00001	"		0	0	
Controls without immune serum.			At 24 hrs.		
0.000001			+		
0.0000001			+		
Dextrose blood broth control.					
0.0000001			+ (in 12 hrs.).		
Dextrose blood agar plate.					
0.0000001			91 colonies.		
"			80 "		

* −, not examined.

Experiment 2.—(Table II.) The tubes were agitated at a rate of 24 revolutions and 20 oscillations per minute continuously until times of culture, as indicated in the table. Parallel sets of tubes were cultured in dextrose blood broth and dextrose blood agar plates.

The results of this experiment (Table II) show that after as short a period as 12 hours pneumococci could not be recovered in culture from those tubes containing less than 0.001 cc. of the standard suspension. Culture into dextrose blood agar plates gave approximately the same result as did the broth cultures. If anything, the blood broth proved to be a slightly more sensitive medium than the blood agar. Plate controls seeded immediately after the completion of the test with 0.0000001 cc. of the standard suspension yielded 80 and 91 colonies respectively. The blood broth tubes showed abundant macroscopic growth in 12 hours. The greater degree of growth inhibition in this experiment, as contrasted with Experiment 1, is due to the employment of a larger quantity of immune serum.

While the culture media employed in the above two experiments for the detection of viability proved to be of a highly suitable nature for growth of the pneumococcus, these media, after all, are artificial and it was felt that before non-cultivability of the pneumococci (following their sojourn in the immune serum-normal serum-leucocyte mixture) could be taken to indicate death of the organism, a more natural culture medium should be used. In Experiment 3 the contents of the serum-leucocyte tubes were injected into field mice, which animals had been found to be very susceptible to the strain of pneumococcus used. 0.0000001 cc. of the standard suspension injected into mice weighing 20 gm. killed regularly in 24 to 36 hours.

Experiment 3.—(Table III.) Tubes were agitated at a rate of 12 revolutions and 10 oscillations per minute for 2½ hours, then the rate was reduced to 7 to 8 revolutions and 6 to 7 oscillations until 12 hours had elapsed, when agitation was stopped and the tubes left undisturbed in the incubator for the remainder of the 24 hours. At this time the contents of the tubes were examined microscopically, then injected into field mice. One set was also cultured in the usual dextrose blood broth. The mouse controls were injected immediately after the test had been assembled. The mice were observed for 12 days.

The employment of mice as the media for testing viability gave essentially the same results as did the dextrose blood broth and blood

TABLE III.

Mouse Inoculation as Test for Viability.

Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + immune serum dilution 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Amount of immune serum.	At 24 hrs.			Culture media.
		Color change.	Pneumococci in stained film.	Growth in culture.	
cc.	*cc.*				
0.01	0.001	0	+	+	Dextrose blood broth.
0.001	"	0	0	0	
0.0001	"	0	0	0	
0.00001	"	0	0	0	
0.000001	"	0	0	0	
0.01	"	0	+	D. 21 hrs.	Field mice. Weight 20 gm.
0.001	"	0	0	S.	
0.0001	"	0	0	"	
0.00001	"	0	0	"	
0.000001	"	0	0	"	
0.001	0.0001	0	0	"	Field mice. Weight 20 gm.
0.0001	"	0	0	"	
0.00001	"	0	0	"	
0.000001	"	0	0	D. 12 hrs.*	
0.0000001	"	0	0	S.	
Control without immune serum.					
0.0000001		++++	+		
Blood broth control.					
0.0000001				+	
Mouse control.					
0.000001		D. 30 hrs.			
0.0000001		" 30 "			

S., survived.

D., died.

* Death not due to pneumococcus infection. No organisms recovered at autopsy.

agar. 0.001 cc. of the standard suspension, after 24 hours sojourn in the immune serum-normal serum-leucocyte mixture, failed to kill the test mice, while the control mouse injected with 0.0000001 cc. of the same suspension died within 30 hours.

Mechanical Factors Affecting the Reaction.

Experiment 4.—(Table IV.) Comparison of slow with rapid agitation for varying periods of time. The tests with the two speeds were carried out on different days but under as nearly identical conditions as possible. It was necessary, of course, to employ a different suspension each day. The possibility of error thus introduced has been minimized by several repetitions of the experiment. A type instance will be found in Table IV. With those tubes agitated at a slow rate, the agitator was run at 5 revolutions and 4 oscillations per minute; the rapid rate was 25 revolutions and 20 oscillations per minute. Periods of agitation are indicated in the table. Those tubes agitated for less than 24 hours were removed from the apparatus and allowed to stand undisturbed in the incubator until time of culture.

The results of this experiment (Table IV) show clearly that speed of agitation affects the degree of growth inhibition. With slow agitation, growth was irregular in those tubes agitated for less than 9 hours; while rapid agitation for only 2 hours elicited the maximum degree of growth inhibition encountered. It is true that continuous slow agitation for a long period produced the same effect as rapid agitation for a brief period. The conclusion might be drawn from the outcome of this test that agitation for 72 hours produced the optimum degree of inhibition, since in those tubes agitated for this length of time growth failed to occur with quantities of pneumococci less than 0.01 cc. However, subsequent tests have not shown this to be the case. Similar slight variations have been found to occur occasionally, quite independent of time of agitation or any other discoverable factors. In most of the tests in which 0.001 cc. of immune serum was employed, inhibition occurred regularly in all those tubes containing less than 0.01 cc. of the standard suspension. In some experiments, however, only the tubes with less than 0.001 cc. of organisms showed no growth. Occasionally in a single test certain tubes will show inhibition above 0.01 cc. and others above 0.001 cc. of the standard suspension. These variations may depend on differences between the serum and leucocytes of the individual animals used. Altogether the tests have

TABLE IV.

Time and Rate of Agitation.

Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + immune serum dilution 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Amount of immune serum.	Time of agitation.	Slow agitation.		Rapid agitation.	
			At 24 hrs.		At 24 hrs.	
			Pneumococci in stained film.	Growth in culture.	Pneumococci in stained film.	Growth in culture.
cc.	*cc.*	*hrs.*				
0.01	0.001	2	+		+	
0.001	"		0	+	0	+
0.0001	"		0	0	0	0
0.00001	"		0	0	0	0
0.01	"	3	+		+	
0.001	"		0	+	0	+
0.0001	"		0	+	0	0
0.00001	"		0	0	0	0
0.01	"	6	+		+	
0.001	"		0	+	0	+
0.0001	"		0	+	0	0
0.00001	"		0	+	0	0
0.01	"	9	+		+	
0.001	"		0	+	0	+
0.0001	"		0	0	0	0
0.00001	"		0	0	0	0
0.01	"	12	+		−	−
0.001	"		0	+	−	−
0.0001	"		0	0	−	−
0.00001	"		0	0	−	−
0.01	"	24	+		0	+
0.001	"		0	+	0	+
0.0001	"		0	0	0	0
0.00001	"		0	0	0	0
			At 72 hrs.		At 72 hrs.	
0.01		72	+		+	
0.001			0	0	0	0
0.0001			0	0	0	0
0.00001			0	0	0	0
Controls without immune serum.			At 24 hrs.	At 24 hrs.		
0.000001		72	+		+	
0.0000001			+		+	

shown that after a certain minimum period of agitation, which should be not less than 6 hours, further agitation does not make any difference in the results.

Rate at Which Bactericidal Action Occurs.

Tests were next carried out to determine if possible the time required for destruction of the pneumococci in the immune serum-normal serum-leucocyte mixtures.

TABLE V.

Rate at Which Bactericidal Action Occurs.

Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + immune serum dilution 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Amount of immune serum.	Culture. At hrs.					
		1	3	6	9	12	72
cc.	*cc.*						
0.001	0.001	+	+	+	+	+	
0.0001	"	+	+	+	+	+	
0.00001	"	+	0	0	0	0	
0.000001	"	0	0	0	0	0	
0.01	"						+
0.001	"						0
0.0001	"						0
0.00001	"						0
Controls without immune serum.		Pneumococci in stained film at 72 hrs.					
0.000001				+			
0.0000001				+			

Experiment 5.—(Table V.) Tubes were agitated continuously at a rate of 20 revolutions and 16 oscillations per minute. At times indicated in the table, tubes were removed from the agitator and the contents cultured in dextrose blood broth.

Cultures made of those tubes agitated and incubated for only 2 hours yielded growth with all except the highest dilution, 0.000001 cc. of the standard suspension. Since the culture from the control tube

at this dilution was positive, it follows that beginning destruction of pneumococci had occurred at this time. After 3 hours, well marked bactericidal action was evident. From this time on until after 12 hours no further increase in the degree of growth inhibition occurred. Those tubes agitated for 72 hours showed a considerably greater inhibition of growth. In Table II it is seen that maximum inhibition of pneumococcus growth does not occur until after 24 hours incubation in the serum-leucocyte mixture. As was stated in the previous communication failure of the pneumococci to grow in highly sensitive culture media after incubation for 2 to 3 hours in the serum-leucocyte mixture does not constitute absolute proof that bactericidal action has taken place by this time but is strongly indicative that such is the case.

Quantitative Action of Leucocytes.

Experiment 6.—(Table VI.) The leucocyte suspension used contained as usual 0.05 cc. of red blood cells. It was employed in quantities of 0.1 cc. except in the first three tubes, which received 0.2 cc. of the suspension containing 10,000 leucocytes per c.mm. Those suspensions containing less than 10,000 leucocytes per c.mm. were prepared by diluting the original (10,000) suspension in gelatin-Locke's solution. Agitation was maintained at a speed of 25 revolutions and 20 oscillations throughout the duration of the experiment.

It is seen in Table VI that a quantitative relationship exists between the number of leucocytes in the suspension used and the degree of growth inhibition brought about. The quantity of leucocytes chosen at first arbitrarily was found to produce the maximum effect. Doubling this quantity produced no further increase in growth inhibition, whereas diminishing the number to 1,000 leucocytes per c. mm. caused a marked reduction in inhibition of growth, and a further decrease to 100 leucocytes per c.mm. resulted in a complete disappearance of this action. Repetitions of the experiment gave similar results.

Quantitative Action of Immune Serum.

Experiment 7.—(Table VII.) The only variable in this experiment was the quantity of immune serum added. Dilutions of immune serum varying from 1 : 5 to 1 : 50,000 were employed, representing quantities of the undiluted serum from 0.1 to 0.00001 cc. The figures in the table indicate the actual dilution of the immune serum in the serum-leucocyte mixture. The rate and time of agitation were the

same as in Experiment 6. Microscopical examination and culture of the tubes were made at 72 hours.

It was found that maximum growth inhibition was produced with dilutions of immune serum as high as 1:500. A concentration of 1:5 failed to produce any greater effect. However, as higher dilutions

TABLE VI.

Quantitative Action of Leucocytes.

Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + immune serum dilution 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Amount of immune serum.	No. of leucocytes per c.mm.	Color change. At hrs. 24	48	72	At 72 hrs. Pneumococci in stained film.	Growth in culture.
cc.	*cc.*						
0.01	0.001	20,000	++++			+	
0.001	"	"	0	0	±	0	0
0.0001	"	"	0	0	±	0	0
0.01	"	10,000	++++			+	
0.001	"	"	0	0	0	0	0
0.0001	"	"	0	0	0	0	0
0.00001	"	"	0	0	0	0	0
0.01	"	5,000	++++			+	
0.001	"	"	0	++++		+	
0.0001	"	"	0	0	0	0	0
0.00001	"	"	0	0	0	0	0
0.01	"	1,000	++++			+	
0.001	"	"	++++			+	
0.0001	"	"	++++			+	
0.00001	"	"	0	0	0	0	0
						At 24 hrs.	
0.001	"	100	++++			+	
0.0001	"	"	++++			+	
0.00001	"	"	++++			+	
0.000001	"	"	++++			+	
Controls.						At 7 2 hrs.	
0.000001	0	10,000	++++			+	
0.0000001	0	"	++++			+	
"	0.001	0	++++			+	

TABLE VII.

Quantitative Action of Immune Serum.

Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + immune serum dilution 0.1 cc. + pneumococcus suspension 0.1 cc.

Amount of standard suspension.	Dilution of immune serum.	Color change. At hrs. 24	48	72	At 72 hrs. Pneumococci in stained film.	Growth in culture.
cc.						
0.1	1:5	++++			+	
0.01	"	0	0	++++	+	
0.001	"	0	0	0	0	0
0.1	1:50	++++			+	
0.01	"	0	0	+	+	
0.001	"	0	0	0	0	0
0.01	1:500	++++			+	
0.001	"	0	0	0	0	0
0.0001	"	0	0	0	0	0
0.00001	"	0	0	0	0	0
0.01	1:1,000	++++			+	
0.001	"	0	+++	++++	+	
0.0001	"	0	0	0	0	0
0.00001	"	0	0	0	0	0
0.01	1:2,000	++++			+	
0.001	"	0	++++		+	
0.0001	"	0	0	0	0	0
0.00001	"	0	0	0	0	0
0.001	1:4,000	++++			+	
0.0001	"	0	0	0	0	0
0.00001	"	0	0	0	0	0
0.000001	"	0	0	0	0	0
0.001	1:8,000	++++			+	
0.0001	"	0	++++		+	
0.00001	"	0	0	0	0	0
0.000001	"	0	0	0	0	0
0.0000001	"	0	0	0	0	0
0.0001	1:16,000	++++			+	
0.00001	"	++++			+	
0.000001	"	++++			+	
0.0000001	"	0	0	0	0	0

TABLE VII— *Concluded.*

Amount of standard suspension.	Dilution of immune serum.	Color change. At hrs. 24	48	72	At 72 hrs. Pneumococci in stained film.	Growth in culture.
0.00001	1:32,000	++++			+	
0.000001	"	++++			+	
0.0000001	"	0	0	0	0	0
0.00001	1:50,000	++++			+	
0.000001	"	++++			+	
0.0000001	"	++++			+	
Controls without immune serum.						
0.000001		++++			+	
0.0000001		++++			+	

of the immune serum were reached (Table VII), the degree of growth inhibition decreased steadily until at a dilution of 1:50,000 this property of the serum-leucocyte mixture disappeared. It is of interest to note that even with an immune serum dilution of 1:32,000, there was a definite, if slight, growth-inhibitory and bactericidal action.

Comparison of Immune Serum Action in Vitro with Protective Action in Mice.

Having found that the addition of antipneumococcus serum to a mixture of the serum and leucocytes of a susceptible animal, the rabbit, conferred on this mixture growth-inhibitory and bactericidal properties for the pneumococcus, and furthermore that the effect of the immune serum was quantitative in nature, it remained to determine whether the action of the immune serum in the test-tube was comparable to its protective function in the animal body. In order to test this point it was necessary to compare two immune sera of different potencies, for their *in vitro* and *in vivo* activity.

Experiment 8.—(Table VIII.) The immune serum, Horse 1, was the therapeutic antipneumococcus serum, Type I, supplied by the New York State Department of Health, dated 1919. Immune serum, Rabbit 4, was one prepared in the laboratory several months previously, according to the method used by Cole and Moore.[3]

[3] Cole, R., and Moore, H. G., *J. Exp. Med.*, 1917, xxvi, 537.

TABLE VIII.

Comparison of Immune Serum Action in Vitro with Protective Action in Mice.

Normal rabbit serum 0.2 cc., rabbit leucocyte suspension 0.1 cc., immune serum dilution 0.1 cc., pneumococcus suspension 0.1 cc.

(*a*) *Growth Inhibition.*

	Amount of standard suspension.	Dilution of immune serum.	Color change. At hrs. 24	48	72	At 72 hrs. Pneumococci in stained film.	Growth in culture.
	cc.						
Immune serum, Horse 1.	0.000001	1:250	0	0	0	0	0
	"	1:375	0	0	0	0	0
	"	1:500	0	0	0	0	0
	"	1:1,250	0	0	0	0	0
	"	1:2,500	0	0	0	0	0
	"	1:3,750	0	0	0	0	0
	"	1:5,000	0	0	0	0	0
	"	1:12,500	+	++++		+	
Immune serum Rabbit 4.	"	1:50	0	0	0	0	0
	"	1:125	0	0	0	0	0
	"	1:250	0	0	0	0	0
	"	1:375	0	0	0	0	0
	"	1:500	0	0	0	0	0
	"	1:1,250	0	0	0	0	0
	"	1:2,500	0	+	++++	+	
	"	1:3,750	+	++++		+	
	Control without immune serum.						
	0.000001		++++			+	

(*b*) *Mouse Protection.*

	Amount of standard suspension.	Amount of immune serum.	Result.
	cc.	*cc.*	
Immune serum, Horse 1.	0.4	0.2	S.
	0.2	"	"
	0.1	"	"
	0.01	"	"
	0.001	"	"
	0.0001	"	"
	0.00001	"	"
Immune serum, Rabbit 4.	0.2	"	D. 12 to 15 hrs.
	0.1	"	" 12 " 15 "
	0.01	"	S.
	0.001	"	"
	0.0001	"	"
	0.00001	"	"
	Control without immune serum.		
	0.0000001		D. 21 hrs.

This serum was inactivated at 56° for ½ hour before use. The tubes were agitated continuously for 72 hours. The mouse protection test was performed on another day with a different suspension. Chinese field mice weighing 20 gm. were used for this purpose. The mice were observed for a period of 10 days after inoculation.

The result of the mouse protection test (Table VIII) showed that the immune horse serum was much more potent than the immune rabbit serum. Mice injected with 0.2 cc. of the horse serum survived a dose of 0.4 cc. of a suspension of pneumococci, 0.0000001 cc. of which killed the control mouse in 21 hours, while mice receiving the rabbit serum failed to survive an infecting dose of 0.1 cc. of the same suspension. A similar difference between the potency of the two sera was found in the outcome of the growth inhibition test. A dilution of the immune horse serum as high as 1:5,000 was effective in preventing the growth of 0.000001 cc. of the standard suspension of pneumococci, whereas dilutions of the immune rabbit serum above 1:1,250 failed to cause growth inhibition of the same amount of pneumococcus suspension. A second test was carried out, with two immune sera of greater difference in their protective action in mice.

Experiment 9.—(Table IX.) The immune serum, Horse 2, was the serum used throughout the majority of the previous experiments. Immune serum, Rabbit 2, was one prepared similarly to immune serum, Rabbit 4, but it had been obtained 2½ years prior to this experiment. The tubes were agitated at a rate of 5 to 6 revolutions and 4 to 5 oscillations per minute continuously for 24 hours, when they were examined microscopically and cultures made. The mouse protection test was carried on at the same time as the growth inhibition test, and the mice were inoculated with the same pneumococcus suspension. Field mice were used as in Experiment 8.

In this experiment the immune serum, Horse 2, showing well marked protective properties for mice, was found to produce marked inhibition of growth in the rabbit serum-leucocyte mixture; while immune serum, Rabbit 2, which afforded only feeble protection to mice against pneumococcus infection, was found to exert a correspondingly weak effect in the growth inhibition test.

The normal serum of animals naturally resistant to pneumococcus infection such as the cat and the dog, failed, even in high concentration, to bring about growth inhibition in the rabbit serum-leucocyte mixture.

TABLE IX.

Comparison of Immune Serum Action in Vitro with Protective Action in Mice.

Normal rabbit serum 0.2 cc., rabbit leucocyte suspension 0.1 cc., immune serum dilution 0.1 cc., pneumococcus suspension 0.1 cc.

(*a*) *Growth Inhibition.*

	Amount of standard suspension.	Dilution of immune serum.	At 24 hrs.		
			Color change.	Pneumococci in stained film.	Growth in culture.
	cc.				
Immune serum, Horse 2.	0.000001	1:500	0	0	0
	"	1:1,000	0	0	0
	"	1:2,000	0	0	0
	"	1:4,000	0	0	0
	"	1:8,000	+*	0	0
	"	1:16,000	++++	+	
Immune serum, Rabbit 2.	"	1:50	0	0	0
	"	1:100	++++	+	
	"	1:200	++++	+	
	"	1:500	++++	+	
	"	1:1,000	++++	+	
	"	1:2,000	++++	+	
	"	1:4,000	++++	+	
	"	1:8,000	++++	+	
	Controls without immune serum.				
	0.000001		++++	+	
	0.0000001		++++	+	

(*b*) *Mouse Protection.*

	Amount of standard suspension.	Amount of immune serum.	Result.
	cc.	*cc.*	
Immune serum, Horse 2.	0.2	0.2	S.
	0.1	"	"
	0.01	"	"
	0.001	"	"
Immune serum, Rabbit 2.	0.1	"	D. 19 hrs.
	0.01	"	" 104 "
	0.001	"	" 48 "
	0.0001	"	S.
	0.00001	"	"
	Controls without immune serum.		
	0.000001		D. 36 hrs.
	0.0000001		" 36 "

* Hemolysis.

Attempts to use citrated whole blood in place of the washed leucocytes and serum have thus far proved unsuccessful.

DISCUSSION.

The parallelism found to exist between the *in vitro* and the *in vivo* action of the immune sera tested, would indicate that the effect of the immune serum in the serum-leucocyte mixture is at least comparable if not similar in nature to that produced in the serum-protected animal. It has been shown that in the test-tube death of the pneumococci is brought about by the combined action of the immune serum, normal serum, and leucocytes.[4] Whether or not destruction of the pneumococci occurs entirely within the leucocytes has not been determined, but it is certain that the leucocytes play an essential rôle in the process since the number of organisms killed was found to be dependent on the quantity of leucocytes present.

The absence of any appreciable antitoxic or growth-inhibitory effect on the part of the immune serum itself was indicated by the finding that after the addition of a certain minimum amount of immune serum —presumably the quantity required for complete opsonization of all the pneumococci present—a further addition failed to produce an increase in the number of pneumococci killed. The part agglutination may play in the reaction was not investigated further than to note that agglutination could not be detected in those tubes containing the higher dilutions of immune serum which were nevertheless very effective in causing growth inhibition and death of the implanted pneumococci.

Of the methods used to test the potency of antipneumococcus serum, the determination of its protective action in mice has proved by far the most satisfactory, since this gives direct evidence of the power of the immune serum to assist the normal defense mechanism to kill the invading pneumococci. The agglutination titer, while of value in estimating the strength of the freshly obtained serum, is at best an indirect measure of its action in the body and cannot be used to test sera that have stood for some months, as the agglutinins tend to

[4] A further communication will deal with the part played by the fresh serum in this reaction.

diminish progressively and not infrequently disappear entirely from an immune serum which is still highly potent. A test-tube reaction, in which the essential immune properties of the serum could be determined quantitatively, would have some advantages over an animal test, in the way of eliminating certain disturbing effects such as variations in individual resistance, the occurrence of secondary infection, etc., as well as shortening considerably the time required for the test. The growth inhibition test with a rabbit serum-leucocyte mixture has given results indicating a usefulness for this purpose. Repeated tests on the same lot of serum, by means of the method of progressive serum dilution, with a fixed quantity of pneumococci, have given surprisingly constant results; *i.e.*, the immune serum action has disappeared in each test at approximately the same dilution. Whether the growth inhibition test is as delicate an indicator of the strength of the immune serum as mouse protection it is impossible at present to say.[5]

SUMMARY.

Using the method described in an earlier publication for testing the growth-inhibitory and bactericidal activity of serum-leucocyte mixtures for the pneumococcus, a study has been made of the action of antipneumococcus serum added to the serum and leucocytes of the rabbit, which alone lack the power to inhibit pneumococcus growth. It was found that the addition of relatively small quantities of immune serum to a mixture of rabbit serum and washed rabbit leucocytes conferred on this mixture marked growth-inhibitory and pneumococcidal powers. The effect of the immune serum was found to be quantitative in nature. Beyond a certain point progressive dilution of the serum resulted in a corresponding decrease in the degree of growth inhibition, but this property of the immune serum did not disappear until a high dilution was reached. The leucocytes were also found to exert their effect quantitatively. Within certain limits the number of pneumo-

[5] Subsequent experiments, in which certain modifications in technique—described in Paper IV—were employed, showed that the immune serum activity could be demonstrated in even higher dilutions than were effective in the above tests.

cocci killed was dependent on the quantity of them present. Tests comparing the action of immune serum in the rabbit serum-leucocyte tubes with their protective action in mice showed a close parallelism between these two effects; that is to say, an immune serum showing marked protective properties for mice was likewise effective in high dilution in the *in vitro* test, and *vice versa*.

STUDIES ON PNEUMOCOCCUS GROWTH INHIBITION.

IV. A Simplified Agitator for Growth Inhibition Tests with Serum-Leucocyte Mixtures; and Certain Modifications in the Technique of the Test.

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Plate 19.

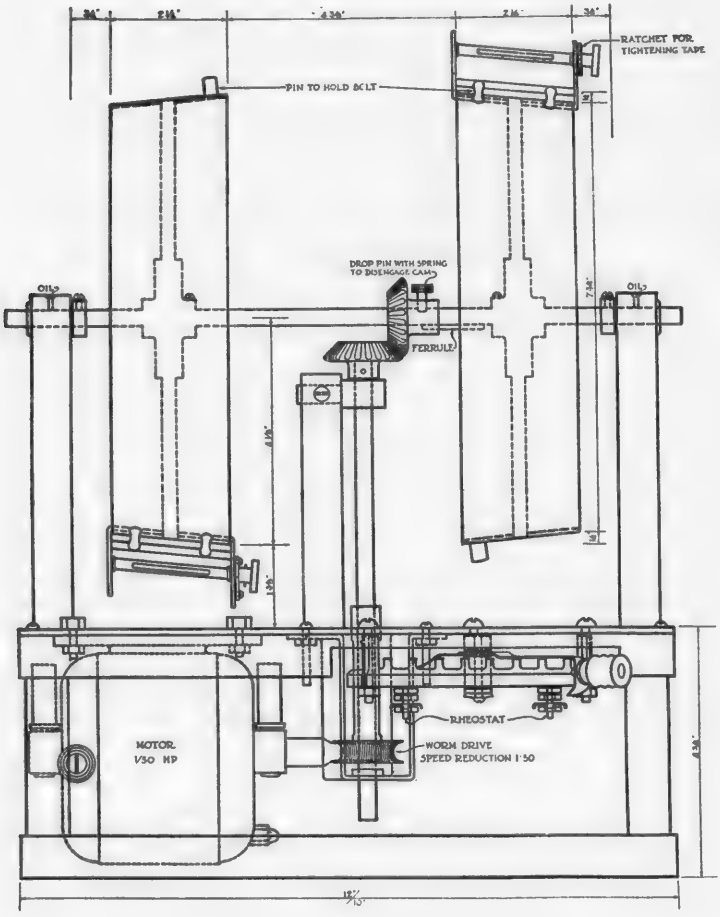
(Received for publication, June 4, 1924.)

Since the publication of the second study of this series[1] a much simpler and more compact agitating apparatus has been devised. Several changes have also been made in the technique of the growth inhibition test which have eliminated occasional irregularities in the outcome that had necessitated discarding the results of the individual experiment. Furthermore, it has been found that some of the test ingredients may be prepared the day before, thus shortening considerably the time required for the actual setting up.

Agitator.

The agitating apparatus pictured in Text-fig. 1 and Fig. 1 is made entirely of metal. It consists of two brass wheels with rims made in the form of truncated cones. The outer rim is ½ inch smaller than the inner. A 1/50 h.p. motor, fixed to the base plate, drives the apparatus by means of a vertical shaft with a worm gear which gives a speed reduction of 1/50. A rheostat makes possible further slowing of rotation rate. The small gear wheel on the main (horizontal) shaft can be disengaged by pulling out the drop pin. The leather tube belt, cut from a pattern, is secured on the wheel by means of three pins and a small perforated shaft with a ratchet which takes up the slack tape and holds the belt firmly to the rim surface. In order to avoid undue strain on the gearing, the motor is started with the wheels free-running. Rotation is then initiated by hand and the sliding

[1] Robertson, O. H., and Sia, R. H. P., *J. Exp. Med.*, 1924, xxxix, 219.



TEXT-FIG. 1. Diagram of agitator drawn to scale.

gear pushed into lock while moving. The apparatus in operation causes no appreciable vibration or heating of the incubator.

The rotation and oscillation given to the contents of the small tubes by this agitator bring about just as effective a mixing as when these two motions are carried out at different rates of speed, for results of duplicate tests, run simultaneously, with the two types of apparatus, are identical. A speed of 20 to 25 revolutions per minute is usually employed.

Modifications in Technique.

Pneumococcus Suspension.—In experiments with rabbit serum and leucocytes, it has occasionally happened that the control serum-leucocyte tubes, seeded with 0.0000001 cc. of the standard suspension (of high virulence for rabbits) grew out very slowly or failed to show any growth in spite of the fact that the dextrose blood broth controls grew well and blood agar plates yielded the usual number of pneumococcus colonies. The results of such a test were, of course, worthless. It seemed evident that the pneumococci had been injured sufficiently during suspension to render them susceptible to destruction by the normal rabbit serum-leucocytes although their ability to grow in a highly favorable medium was unimpaired. Investigation of the cause of this injury proved it due to prolonged suspension of the organisms in high dilution in the gelatin-Locke's solution.[2] When the pneumococcus suspension was delivered into the serum-leucocyte tubes soon after the dilution had been completed, the control tubes always showed abundant growth after 15 to 18 hours incubation. It was found possible to lessen greatly or to prevent entirely this deterioration of the pneumococcus suspension by adding 2 per cent of a M/15 balanced phosphate mixture, pH 7.8, to the gelatin-Locke's solution. However, it would seem wisest, even with phosphate added to the suspension fluid, to distribute the organisms into the serum-leucocyte tubes as soon as possible after the dilutions are completed. If a delay is necessary, the pneumococci should be allowed to remain in concentrated suspension. Once the organisms are seeded into the serum-leucocyte mixture, further standing for several hours at room temperature does not appear to retard growth after incubation begins.

Leucocytes.—A series of experiments on keeping washed rabbit leucocytes for varying lengths of time before use has shown that when suspended in their own serum, the leucocytes may be preserved in the ice box for a period as long as 48 hours without showing any deterioration in their activities as concerns pneumococci. In one test leucocytes obtained 5 days previously gave the same results

[2] Controls on the reaction of the suspension showed that injury to the pneumococci could not be attributed to variations in the H ion concentration. It is probably due to a toxic salt action.

as freshly prepared ones. Results with cat leucocytes are less conclusive and will need further investigation. The above finding with rabbit leucocytes makes it possible to prepare the normal serum-leucocyte mixture the day before the test is to be set up, which is a considerable advantage if the experiment includes a large number of tubes. In order to prevent the very occasional formation of fibrin in the gelatin-Locke's cell suspension, it has been found advisable to wash the leucocytes and red blood cells twice in gelatin-salt solution. If the cells are to be kept, the supernatant fluid is completely removed after centrifugation in gelatin-Locke's solution. The red blood cells are next added to the leucocytes in the usual amount. Then a quantity of the serum of the same animal sufficient to provide for the number of serum-leucocyte tubes required in the test, with 0.2 cc. of serum for each tube, is well mixed with the cells and the whole placed in the ice box. To keep the total fluid volume the same as that regularly used, an amount of gelatin-Locke's solution equivalent to 0.05 cc. for each serum-leucocyte tube is added to the serum-leucocyte mixture before it is distributed into the small tubes.

Normal Serum.—Numerous observations have shown that, in general, immune serum is effective in higher dilutions when used with normal serum and leucocytes obtained from the same animal. Hence fresh serum sufficient for the entire test should be secured, if possible, from the animal providing the aleuronat exudate.

Small Glass Tubes.—In our earlier work little attention was paid to the quality of glass in the small tubes used. However, the not infrequent occurrence of early hemolysis in a few or many tubes of an individual test, sometimes intense enough to disturb the results, pointed to impurities in the glass as a possible cause of blood cell injury. The use of Pyrex tubes has entirely eliminated this source of error.

SUMMARY.

A simplified and compact agitator for growth inhibition tests with serum-leucocyte mixtures has been described.

Several modifications have been made as well in the technique of the test, which have eliminated occasional irregularities that

necessitated discarding the results of the individual experiment. Such irregularities were found to be due chiefly to injury of the pneumococci brought about by prolonged suspension in gelatin-Locke's solution which resulted in failure of the organisms to grow in the control serum-leucocyte tubes. This deterioration of the pneumococcus suspension may be greatly lessened or entirely prevented by the addition of a small quantity of a balanced phosphate mixture to the gelatin-Locke's solution. The use of small tubes made of Pyrex glass has also eliminated the former, not infrequent, occurrence of early hemolysis which was sometimes intense enough to disturb the results of the test.

It has been found that washed rabbit leucocytes, suspended in their homologous serum, may be kept in the ice box for as long a period as 2 days without showing any apparent diminution of their functional activity in the serum-leucocyte test.

EXPLANATION OF PLATE 19.

FIG. 1. Simplified agitator used for growth inhibition tests with serum-leucocyte mixtures.

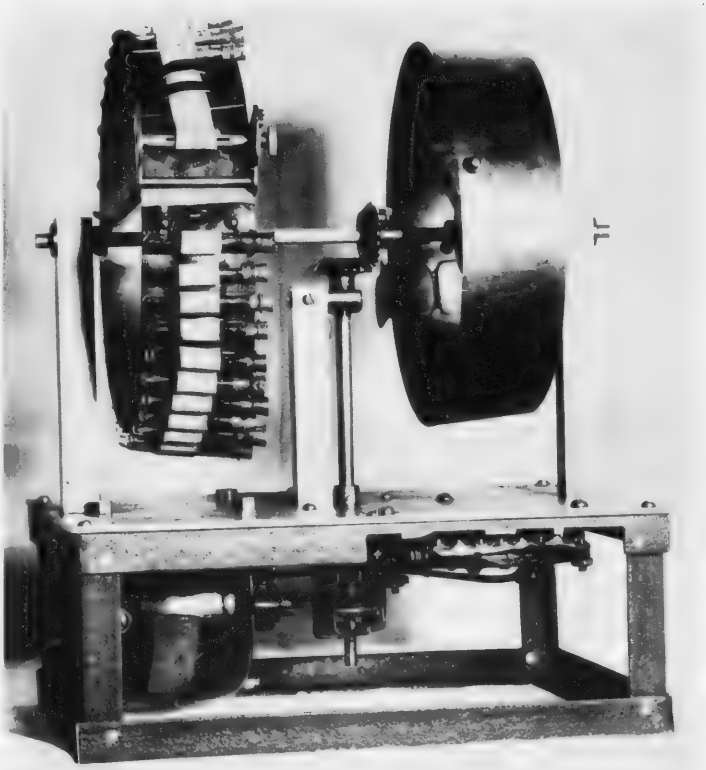


FIG. 1.

(Robertson, Woo, and Cheer: Pneumococcus growth inhibition. IV.)

STUDIES ON THE BIOLOGY OF STREPTOCOCCUS.

IV. The Occurrence of Streptococcus scarlatinæ in Convalescence and in the Complications of Scarlet Fever.

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Recent studies of the etiology of scarlet fever have demonstrated conclusively that a group of hemolytic streptococci which possess specific antigenic and peculiar toxic properties is associated with the angina of scarlet fever.

Strains of streptococci from cases of scarlet fever have been agglutinated by Dochez and Bliss (1–3), Tunnicliff (4, 5), Gordon (6), and by Stevens and Dochez (7), who have found that a streptococcus giving characteristic agglutination with scarlatinal immune sera is present in from 65 to 85 per cent of all cases of scarlet fever during the acute stages of the angina. These streptococci have not only been cultured from the throat during the acute stages of the disease but have been obtained from wounds in cases of surgical scarlet fever, and have been recovered from milk which was apparently responsible for milk-borne epidemics. Tunnicliff and Bliss have been able to obtain streptococci which agglutinated with their sera from convalescent cases of scarlet fever as well as from the attendants in scarlet fever pavilions. Since these agglutination reactions have been carried out, Dick and Dick (8) have demonstrated that the characteristic rash and angina may occur in a certain percentage of people if the fauces are inoculated with pure cultures of streptococci obtained from scarlatinal sources. More recently Dochez and Sherman (9, 10) have produced a serum of distinct therapeutic value by the immunization of horses with living streptococci. This serum not only causes the rash to disappear when injected intramuscularly but regularly induces local blanching if injected intracutaneously within 48 hours after the appearance of the rash (11). Dick and Dick (12) have since obtained skin reactions with diluted filtrates of streptococcus cultures in a certain percentage of individuals who have not previously had scarlet fever. Recent convalescents from scarlatina do not show this reaction. The reaction is neutralized by serum from cases of convalescent scarlet fever and is absent in persons who have been given subcutaneous

inoculations of filtrate. According to Zingher (13), who has repeated their work, the reaction is analogous in many respects to the intradermal Shick test.

The identification of this group of hemolytic streptococci which are so intimately associated with scarlet fever is of great epidemiologic importance. It is possible that they may be identified by means of skin reactions with filtrates and specific neutralization with scarlatinal serum, but the identification of a great number of strains in this manner would be an impractical procedure. At the present time the most practical method by which we may identify this group of hemolytic streptococci associated with scarlet fever is by means of the agglutination reaction. In the literature reviewed in the previous paragraph, Bliss and Tunnicliff point out that streptococci which agglutinate with scarlatinal immune sera occasionally occur in the throats of convalescent cases. Bliss is of the opinion that in the majority of instances streptococci disappear from the throat at about the end of the 2nd week. He has found, however, that they may persist over much longer periods and that carriers may be responsible for small epidemics of contact cases. Since in all probability a number of sporadic cases of scarlatinal infection which occur are return cases resulting from contact with recent cases of scarlet fever, the occurrence of these agglutinable strains of streptococci in the throat and in the complications late in convalescence is of importance. We have employed the agglutination reaction for further study of the distribution of these streptococci under these conditions.

Materials and Methods.

During the winter of 1922–1923 we were able to obtain cultures from a series of patients at Willard Parker Hospital. These cultures were obtained that we might study the occurrence of streptococci in the acute cases and in complications in scarlatina, and determine the percentage of cases in which streptococci were still present at the end of the prescribed period of isolation.

The cultures on the acute cases were obtained between the 3rd and 6th days of illness and were made in the usual manner on blood agar. Rabbits were inoculated with the first five strains isolated and with a Baltimore type scarlatinal strain which

we were able to obtain from Bliss, until their sera gave a 4 plus agglutination reaction with the antigenic strains in dilutions of 1:1,280. The animals were inoculated with increasing amounts of whole broth culture over periods of 4 days with intervals of 4 days between the courses of inoculation. After the fourth course the sera were usually suitable for agglutination. The actual technique of the tests has previously been described by Dochez, Avery, and Lancefield (14), and by Bliss (3). We found that whole broth cultures grown at room temperature were more suitable for agglutination than washed cultures grown at 37.5° (15), but with this exception their technique has been carefully followed. None of the six sera which were prepared agglutinated hemolytic streptococci from pyogenic infections. Many of the cases from which we obtained strains early in the disease were cultured a second time just prior to their discharge from the hospital. The majority of the cultures in the complications were made in cases in which the bacteriology of the throat had been studied.

The results of the agglutination reactions with the strains isolated have been arranged in Tables I to III. The cases have been arranged consecutively in the order in which the cultures were obtained. Table I includes the first forty cases which were cultured between the 3rd and 6th days of the disease. The series of cultures on the convalescent cases, including cultures on many of the positive cases in Table I and additional cases which were cultured when they were discharged from quarantine, is found in Table II. Table III summarizes the agglutination reactions with strains occurring in the purulent exudate in adenitis, rhinitis, and mastoiditis.

Discussion of Bacteriologic Data.

An analysis of the cultures made on the group of acute cases in Table I confirms the work previously reported by other authors. In this group of cases we find hemolytic streptococci present in 87.5 per cent of the throats. This figure is a trifle lower than similar figures previously reported, especially those in reports stating that hemolytic streptococci are to be found in every case of scarlet fever early in the disease. We believe, however, that since all of these cultures were made between the 3rd and 6th days after the onset of the angina, in all probability the throats in the milder cases were already free from the streptococcus. Bliss has already pointed out that in the majority of cases streptococci are not found in cultures made between the 10th and 14th days. 74.3 per cent of these strains isolated were agglutinated

TABLE I.

Agglutination Reactions with Strains of Streptococci from Acute Scarlatinal Throats.

Case No.	Sera.					
	1	II	3	4	5	B1
1	+++	++++	+++		+++	++
2	+++	++	++		+++	+++
3	++	++++	++		++	++++
4	++++	++++	++		+++	+++
5	+++	++++	+++		++	++++
6	++	+++	±		+++	+++
7*						
8*						
9	++	+++	++		++	++
10	++	±	+		+++	++++
11		++	++		+++	++
12	++++	++++	++++		++++	++++
13†						
14	+	+++				++
15	+++	++++	++		+++	±
16	+	++			+	±
17*						
18	++	+++				++
19	++	++			++	+++
20	++	+++			++	+++
21	++	++++			+	+++
22	±	++			++	+++
23	−	±		−	−	−
24	−	−		−	−	−
25	−	++		+	++	+++
26		+++		++		++
27		−		±		−
28		++		+++		+++
29		−		−	−	−
30		−		−	−	−
31*						
32		+++		++	++	+++
33		++		++++	++	±
34†						
35		−		−	±	−
36†						
37		+++		+	++	+++
38*						
39		++		++	++	+++
40		+++		+++	+++	++++

* Streptococci not found.

† Strain granular and inagglutinable.

by type immune sera, so that the cultures from 65 per cent of the cases show hemolytic streptococci definitely belonging to one biological group. Since this study was made, this group of streptococci has been further identified through Type Serum B1, as belonging to a very widely spread group of streptococci always associated with scarlet fever and occurring in widely separated districts. Strains falling in this group have been cultured from milk and have been obtained from cases of scarlet fever occurring in Denmark, Chicago, San Francisco, Baltimore, and in smaller epidemics in other cities in the United States. We have since prepared a toxin apparently identical with the substance obtained by Dick and Dick with representatives of this group.

The strains which were not agglutinated represent by far the minority of streptococci found in these throats. Some of the strains were granular and unsuitable for agglutination reactions. Five of the strains did not agglutinate with any of the sera which we prepared. They may possibly represent a second and less important group of *Streptococcus scarlatinæ*, but from an analysis of Tables II and III a certain number of them are undoubtedly pyogenic strains not primarily associated with the original infection. It is to be expected from the commonness with which streptococci are found in the throat in other infectious diseases, and the frequency of secondary streptococcus infections of the respiratory tract in measles, that secondary infection with non-scarlatinal streptococci would occur in scarlet fever.

After a period of 30 days quarantine, nineteen of the cases in Table I were cultured a second time. All of the nineteen had had streptococci corresponding to the type strains in the earlier throat cultures; eight of the cases still showed streptococci of this group in the cultures made when they left the hospital. Five of the strains recovered were not agglutinated with our sera. We consider this evidence that a mixture of scarlatinal and non-scarlatinal strains occurs in a certain percentage of the cases of scarlet fever. These cases along with nineteen additional convalescents cultured are included in Table II.

In the entire series of thirty-eight cases cultured at the end of the prescribed period of quarantine, nearly half, 47.3 per cent, were carriers of hemolytic streptococci. We were able to agglutinate 55 per cent

TABLE II.

Agglutination of Strains of Streptococci from the Throat in Convalescent Cases of Scarlatina.

Case No.	Day of disease.	Tonsils.	Streptococci present.	Sera.		
				2	4	B1
1	33	+	+	++++	+++	+++
2	34	++	+	±	−	−
4	30	−	−			
6	30	+++	+	++	+++	+++
9	29	++	+	+++		+++
10	35	+	−			
12	32	−	−			
14	30	+	+	++	±	++
15	30	++	−			
16	27	+++	+	++	++	++
18	29	++	+	−		−
19	28	−	−			
21	30	−	+	++++		++++
22	34	++	+	−		−
25	32	+	−			
26	32	+++	+	−		−
32	30	++	+	+++	+++	+++
33	35	++	+	−		−
40	33	++	+	+++	+++	++
41	29	+	−			
42	30	+	+	++++	+++	+++
44	30	+	−			
45	30	−	−			
46	28	+++	+	+++	++	++
47	32	+++	+	−	−	−
48	31	+	−			
50	34	−	−			
51	29	+++	+	++	++	++++
53	30	+	−			
54	32	+++	+	−	−	±
55	27	+	−			
56	28	+	−			
57	27	−	−			
58	30	+	−			
59	28	−	+	±	±	−
60	29	+	−			
61	28	−	−			
62	30	++	++		−	−

of these strains, so that of the total number of cases studied in this group, 29 per cent were carriers of streptococci similar to those isolated

TABLE III.

Agglutination of Strains of Hemolytic Streptococci from Acute Complications of Scarlatina.

Case No.	Source of strain.	Sera.		
		1	4	B1
14	Throat.	++		++
	Otitis.	+++		++
15	Throat.	++++		++
	"	−		−
	Otitis.	+++		+++
23	Throat.	−	−	−
	Otitis.	++	+	++
26	Throat.	+++	++	++
	Rhinitis.	−	±	−
28	Throat.	++	+++	+++
	Adenitis.	++++	++	+++
	Rhinitis.	−	−	−
33	Throat.	++	++++	±
	Adenitis.	++	+++	++
43	"	−		−
49	Mastoiditis R.	−		−
	" L.	−		−
52	"	±		±
63	Otitis.	−		−
64	"	+++		++
65	Empyema.	−		−

from the throats in 65 per cent of the acute cases. Many of the cultures were repeated on 2 or 3 succeeding days. The streptococci were

usually obtained from the crypts of the tonsils. In Table II we have indicated the degree of tonsillar inflammation by plus signs. One plus indicates an apparently normal tonsil and additional plus signs have been used to indicate degrees of inflammation resulting from the angina of the scarlet fever. With the exception of one case, tonsils were present in all of the scarlatinal carriers. This observation is similar to that of Nichols (16) who found that the tonsils were usually the source of continual throat infection in the hemolytic streptococcus carrier state.

The bacteriologic study of the complications which occurred among the 65 cases confirms our belief of secondary invasion with common pyogenic strains of hemolytic streptococci. The results of the throat cultures and cultures from the exudate in these complications are found in Table III. The complications studied occurred late in convalescence. In five of the cases streptococci agglutinating with scarlatinal type serum were present in the throat when the complication occurred. Some of the complications occurring among these cases were apparently due to the scarlatinal strain found in the throat, and in other instances the streptococcus recovered was an extraneous pyogenic strain. In Case 15 an agglutinable and a non-agglutinating strain were obtained in the same throat culture. The complications in the later cases (Nos. 43, 49, 52, 63, 64, and 65) occurred within an interval of a week following the admission of an acute mastoid infection among a group of twenty patients isolated in an annex of the hospital. Only one of the seven strains isolated from these complications could be identified with the group which we had previously obtained. It is our opinion that these non-agglutinating strains occurring in cases from which scarlatinal strains can be isolated represent secondarily invading streptococci, and that the complications in scarlet fever may be caused either by the original scarlatinal strain or by pyogenic strains.

CONCLUSIONS.

1. A group of streptococci which agglutinate with scarlatinal immune sera has been isolated from the throats of 65 per cent of cases of scarlet fever during the 1st week of the disease.

2. Strains of hemolytic streptococci belonging to this group have been isolated from the throats of patients at the termination of quarantine (30 days).

3. Hemolytic streptococci are found most frequently in the throat in convalescent patients in whom the tonsillar inflammation has not entirely subsided.

4. The complications occurring in scarlet fever may be due to the original scarlatinal strain or may be the result of a secondary infection with pyogenic strains of streptococci.

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ALLERGIC IRRITABILITY.

The Formation of Anti-Sheep Hemolytic Amboceptor in the Normal and Tuberculous Guinea Pig.

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GENERAL INTRODUCTION.

Many special cases are now known in which some measure of acquired immunity is transmitted by a mother to her offspring. This transmission is generally believed to be "passive" in its nature, antibodies being transferred either through the placenta or with the milk. The resistance of the young is temporary as would be expected if the immunity depended on a passive transfer of antibodies. The anaphylactic or hypersensitive condition is similarly transmitted by the mother in some instances, appearing as a temporary quality in the young. This sensitization is likewise supposed to be passive, depending on the transfer of a special class of antibodies.

In the course of a discussion of one of the clear cases of the passive transmission of immunity, that against diphtheria toxin, Theobald Smith (1) pointed out that the amount of antibody formed by a given animal was doubtless influenced by the male parent as well as the female: "Though he (*i.e.* the male parent) does not transmit directly any passive immunity, yet there is no evidence to show that he does not equally, with the mother, transmit the capacity for producing antibodies, which capacity . . . . varies much from family to family, quite independently of the treatment."

Later Hagedoorn-La Brand and Hagedoorn (2) described an instance in mice in which resistance to an infection (supposedly by a staphylococcus) is influenced by heredity, control apparently being exercised through one Mendelian character.

Wright and Lewis (3) determined the resistance of pure lines of guinea pigs, developed by continuous brother and sister matings, against infection with the tubercle bacillus and found demonstrable differences, evidently inherited. The

authors consider that several factors are involved. The resistance is under the influence of the male parent equally with the female. The factors seem to be, at least in part, of Mendelian character.

Webster (4) has shown that if mice surviving a controlled epidemic of mouse typhoid infection are interbred their offspring is more resistant to the same and similar infections than is the general stock from which it is derived. Repetition produced a third generation (second selected generation) which was even more resistant. Resistance to mouse typhoid was associated with an increased resistance to poisoning with bichloride of mercury. These experiments make it plain that the resistance dealt with is not wholly specific. They do not show how far, if at all, specifically directed factors are concerned.

In these instances no suggestions appear as to the functional nature of the significant characters,—obviously a question of the utmost interest and one the consideration of which has furnished the background for the experiments presented in this paper.

It is generally believed on the basis of much evidence that the major defenses of the animal body against bacterial invasion involve the activities of the phagocytic cells on the one hand; on the other, the ability to produce and throw into action specific antibodies. We have therefore given precedence to these functions in our consideration of the nature of the factors in question and our ultimate purpose is to see whether variations in antibody production or action, or in the activities of body cells, both as related to the tubercle bacillus, can be correlated with the observed differences in the resistance of the guinea pigs to infection.

Those familiar with the general state of our knowledge of immunity toward tuberculosis will recognize that the possibilities of direct experimental approach to this problem are decidedly restricted. Antibodies against the tubercle bacillus are formed in relatively small amounts and with difficulty by any species. In the case of the guinea pig they have been scarcely more than occasionally demonstrated. It is probably true that as a rule it has not so far been possible to produce in this species any artificial immunity against the tubercle bacillus of comparable degree to the natural variation found by Wright and Lewis (3). The usual procedure of studying the phenomena of the state of acquired immunity with subsequent application to the problem of natural resistance is thus scarcely available to us. For the time being, therefore, our approach has been through

the study of the general cellular and humoral reactions of the animals, in the hope that a fuller knowledge of the peculiarities of these might show correlations with family and that, with the resistance known, suggestions for crucial experiments might appear.

We thought, for example, that it might be possible to determine the capacity of a family of guinea pigs to produce antibodies as a general proposition; that if this were accomplished it might be fair to assume that in so far as antibodies may be influential in tuberculosis they would be produced in accordance with the general scheme, and that then even rather unsatisfactory direct determinations of antibodies against the tubercle bacillus or its products might assume significance according to their relation to the more general result. A considerable number of experiments on this basis made it plain that we had entered upon quite another field to which the classical immunization experiments furnish the general method, but in which our previous knowledge of immunity reactions is insufficient to enable us to foresee results with any degree of accuracy. Our conception of immunity is dominated by the idea of specificity, and properly so, where judged only by the effect produced, either the actual protection attained by passing through a disease, or the recognized reaction products as they become available for examination. But looking at the matter from the view-point of the animal's capacity to produce antibodies, or, more generally, its capacity to be immunized, it becomes a pertinent question whether the mechanisms concerned are specific, wholly, in part, or not at all. Assuming that the mechanisms are highly specialized, can a number of them operate simultaneously at highest efficiency? Or if, on the contrary, the mechanism is common to all, can it be occupied by the first comer to the temporary exclusion of all others? It is to these questions that the experiments here reported relate directly. It has also developed that our knowledge of the reactions of the guinea pig to foreign cells has been incomplete and the observations reported add something to it.

Our title requires a brief explanation. In use, the phrase "capacity to be immunized" has proved cumbersome, nor does it quite accurately reflect the situation, since it leaves the phenomena of anaphylaxis and hypersensitization out of account or represented only by inference. This has been recognized previously and it has been custom-

ary to say that an animal once subjected to a foreign protein, or once vaccinated against a particular microorganism, is rendered allergic, or put into a state of allergy with reference to that protein or that microorganism. The word, it is true, is not so often used because we have most frequently been dealing definitely with either immunity or hypersensitiveness as particular conditions. Here we are trying to define an underlying mechanism and an accurate terminology may prove useful. Academically considered, as a matter of physiology, the production of immunity or hypersensitiveness would appear to involve the conception of stimulation, and the capacity to be stimulated involves the conception of irritability. We are thus led to propose the term "allergic irritability" as a general characteristic of the animal on the basis of which it reacts to stimuli of the antigenic class, whether they be helpful, injurious, or indifferent to bodily health.

EXPERIMENTAL.

Observational Part.—While using the outline somewhat as above presented as a guide we were led by an accidental occurrence to carry through an experiment designed to show whether a preexisting tuberculosis affected the production of antibody for an antigen unrelated to the tubercle bacillus. The result was definite, showing a decided increase in anti-sheep amboceptor production by tuberculous guinea pigs. Certain other experiments were then carried through to control and amplify the first. The procedures and results are herewith presented.

Methods.—Twenty guinea pigs of Family 13 of the inbred stock as developed at the Bureau of Animal Industry and used by Wright and Lewis[1] were inoculated intraperitoneally with $\frac{1}{100}$ mg. of tubercle bacillus Bovine C_2. The same number of animals, of the same family and in general of the same age, were put aside as controls. At the end of 3 weeks all were injected with washed red blood corpuscles of the sheep. They were given 10 cc. of a 20 per cent suspension, one-half the amount intraperitoneally, one-half subcutaneously. On the 33rd day succeeding, the surviving animals were again injected with the same amount of sheep corpus-

[1] The cooperation with Dr. Wright of the Bureau of Animal Industry has continued since the writers became associated with The Rockefeller Institute. We are gratefully indebted to him for breeding stock and for continuous increments of animals belonging to the inbred families.

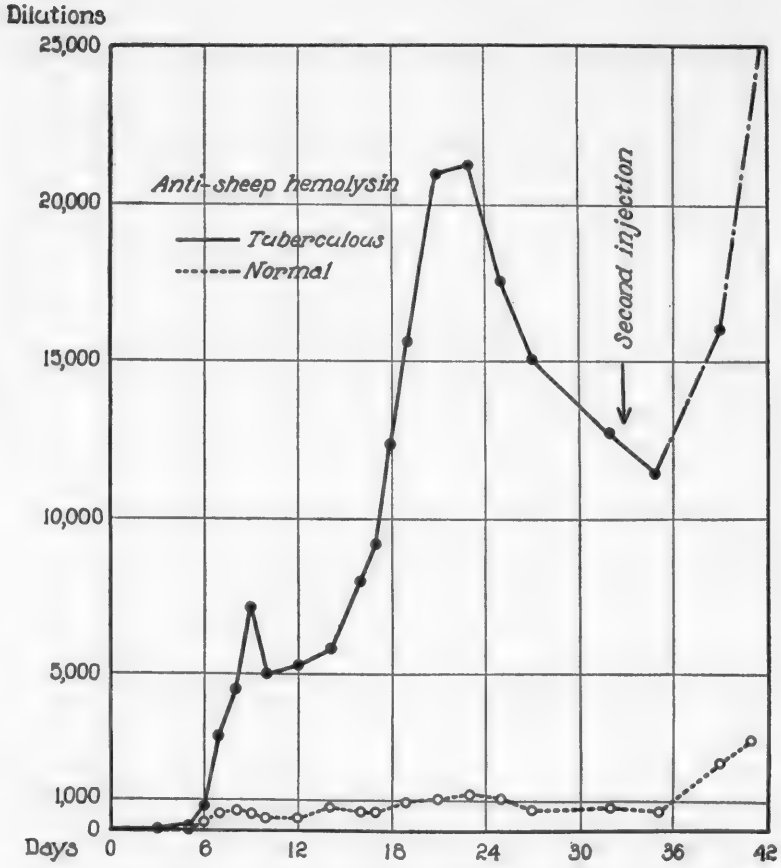

CHART 1. Average amboceptor production of tuberculous guinea pigs (solid line) contrasted with normal guinea pigs (broken line). Tuberculous pigs infected 21 days previous to the zero point of the chart. At zero days all were given 5 cc. of 20 per cent sheep red blood corpuscles intraperitoneally and the same amount subcutaneously. All bled 1 cc. from heart on days 3, 5, 6, 7, 8, 9, 10, 12, 14, 16, 17, 19, 21, 23, 25, 27, 31, 35, 39, 41. A second blood cell injection on the 33rd day.

cles in the same localities. This was successfully accomplished by giving the intraperitoneal portion first in divided doses over a period of several hours, thus avoiding anaphylactoid complications. The animals were bled on the 3rd day following the first injection of corpuscles and at intervals until the 41st day when the experiment was closed.

Bleeding was from the heart. 1 cc. was taken and immediately added to 4 cc. of normal salt solution in small test-tubes. These when clotted were stirred with a wooden applicator and the clot separated, usually by the centrifuge but sometimes by standing in the refrigerator.

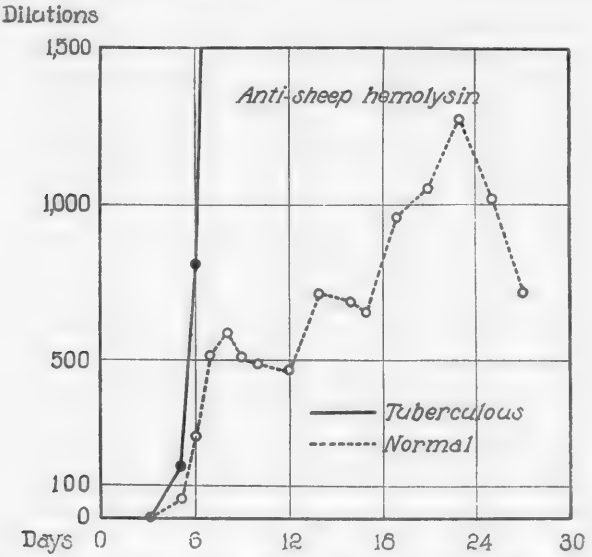

CHART 2. Same observations as Chart 1 but plotted on a larger scale to show that there are significant fluctuations in the curve for the normal animals and that the apical points are coincident with similar points in the tuberculous.

The amboceptor content was determined with these samples in much the usual way. Four units of fresh guinea pig complement (a definite excess) were used. The total volume was 1.1 cc. The amount of red corpuscles was 0.1 cc. of a 10 per cent suspension. The time of incubation in the water bath, at 35–37°C., was ½ hour, and the end-point that of complete hemolysis.

Determinations were made more closely in the case of the less active sera. The original dilution was 1:5 of blood and this was regarded as a dilution of 1:10 of serum. 1:20 was the first dilution tested. Progression was by 10's to 1:100, by 100's to 1:1,000, by 250's to 1:2,000, by 500's to 1:5,000, and by 1,000's beyond that point.

The variable factor in the test itself is the suspension of red corpuscles. This was made to 10 per cent of the whole defibrinated blood, with adjustment to the density of the preceding suspension when necessary. Fresh suspensions were prepared weekly, and it was found that at the end of the week the superficially intact corpuscles had become definitely more susceptible to the hemolytic effect of the serum. Tests of controls and the tubercular series of the same day of bleeding were therefore carried out so far as possible on 1 day. Thus, read vertically, the charts give an accurate comparison between the two groups. The progression of the curves is less accurate, but sufficient checks were done to make certain the day to day changes as observed were essentially correct.

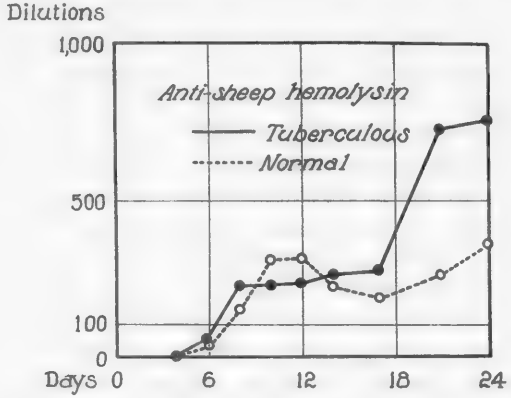

CHART 3. Average amboceptor production of tuberculous guinea pigs contrasted with normal guinea pigs. The infection was made 2 days before the injection of the blood cells, the total amount being the same as in the first experiment. The blood cells were given only subcutaneously. Bled 0.5 cc. from heart on days 4, 6, 8, 10, 12, 14, 17, 21, 24.

Bleeding from the heart involves considerable mortality, at best. The numbers included in the averages are progressively smaller as the experiment proceeds. As a check the curves for the surviving individuals have been developed and found to agree in essential characters with those of the averages.

The results of the experiments are shown in three charts and the legends which accompany them.

DISCUSSION.

Amboceptors Produced by Normal Guinea Pigs.—The curve of normal antibody production has been determined many times in the past and

something approaching a general law has seemed to be apparent. Rabbits, goats, sheep, and the horse have been the animals commonly used for these observations. The results have fallen into two classes.

When living bacteria capable of establishing disease processes in the animal have been used the curve has been irregular and prolonged. Fluctuations in the production of antibodies under these circumstances undoubtedly bear a relationship to the changes of balance between host and parasite as these develop and alter with time.

When the antigen used has been dead material, whether indifferent cells or proteins on the one hand or strong toxins on the other, the curves have shown a sharp rise following the last injection, the peak of the rise occurring from the 6th to the 23rd day. A sharp fall succeeds in the next few days followed by a more gradual decline over a period often very prolonged. The differences in detail among the curves as heretofore determined seem to be related to the antigen used. Thus the maximum for diphtheria antitoxin falls on about the 10th day (Salomonsen and Madsen (5)), that for tetanus antitoxin (Brieger and Ehrlich (6)) about the 20th day whether goats or horses be used for production. In the case of hemolytic amboceptor it is commonly accepted that the peak is on the 9th or 10th day, and that this may be advanced to the 7th or 8th day by injecting the antigen intravenously (Sachs (7)). We find no reference to the guinea pig's having been made the basis for observations on this point.

Our determinations agree among themselves in showing a departure from the accepted type. There is always a peak at about the 9th day followed by a sharp fall. Then instead of a steady decline there is a second rise beginning about the 12th day and continuing to a second maximum about the 22nd day. This again has been followed by a sharp decline. Our observations have not been carried beyond this point. It will be noted that Chart 2 shows a small peak at the 14th day also. This has frequently been seen but is somewhat less regular in its occurrence than the apices of the 9th and 22nd days.

Consideration of the experimental details makes it possible to state that this form of curve is the response to a single injection of sheep red corpuscles in the normal guinea pig (Chart 3), and that the simul-

taneous administration of the cells intraperitoneally and subcutaneously has the same effect as a single injection with respect to the form of the curve (Chart 2; *cf.* Chart 3). The amount of antibody formed is apparently somewhat greater when the cells are so administered (Charts 2 and 3 compared).

It is also plain from our experiments, although not shown in the charts, that heredity, in so far as our material permits its consideration, does not influence the form of the curves. Stock crossbred animals and two inbred families have given similar reactions.

Influence of Infection with the Tubercle Bacillus.—As shown by Chart 1 preexisting tuberculosis greatly increases the amount of amboceptor formed. At the point of maximum production and on the average this difference may be as much as twentyfold. A study of the individual animals in the series showed that no animal in the control series produced as much antibody as the least active of the series with tuberculosis. The differences at these extremes were so much less marked, however, that we would expect occasional overlapping if the experiments were often repeated.

It is to be noted that the form of the curves is not altered (compare Charts 1 and 2). This would seem to suggest that the effect of the tuberculosis is one of stimulation to a normal mechanism, or possibly, that the cellular basis of such a mechanism is hypertrophied, rather than that any wholly new factor has been introduced by the disease.

It is evident that the condition underlying increased amboceptor production in the first experiment was fully established when the blood corpuscles were injected. At this time the omentum was thickened moderately and in some animals was definitely the subject of caseous degeneration. Invasion of the liver and spleen was present, early gray tubercles in moderate numbers being just visible to the naked eye. Toward the close of the experiment the liver and spleen showed advanced tuberculosis. The second experiment (Chart 3) was designed to develop the point in the tubercular infection at which the influence in question becomes manifest. It is seen in this chart at the 14th day; in others, essentially the same and not published, the difference is evident on the 15th to the 17th day. With the

dosage and culture used it appears that the effect on amboceptor production will be invariably present and sustained after the 17th day.

In the first experiment (Chart 1) a second injection of blood cells was given 33 days after the first. This has point in the case of the tubercular animals only. There were but two of these left at the time and the graph is continued with a broken line to indicate that in the late range it has not the same finality as the preceding portion based on larger numbers. It does definitely show that the exaggerated amboceptor production of the 1st month did not exhaust the stimulating effect of the disease. In relation to a second injection the tubercular animals reacted similarly to the normal but on an abnormally high level.

Our experiments thus show that the curve of anti-sheep amboceptor production in the guinea pig is of an unusual type, and that in tuberculous guinea pigs, the form of the curve being essentially unaltered, there is a very marked increase in the amount of antibody produced in response to a given injection of antigen from the time the disease is well established.

We have done some complement fixation reactions with the serum of these animals, using tuberculosis antigens, with the idea that the stimulating action on antibody production might be more or less mutual. The results have been entirely negative. This aspect of the subject is not, however, exhausted thereby, as the conditions are far from optimal for the production of tuberculosis antibodies.

There is a considerable literature relating to the ways and means of influencing antibody production. We find no exact analogy to our experiment, no instance in which preexisting disease is clearly shown to influence the production of reaction products toward another antigen.

Directly applicable is the observation of Thompson (8) that old tuberculin or tuberculin B.E. injected into rabbits 1 or 2 days before the administration of sheep red blood corpuscles increased amboceptor production greatly. Quantitatively his results are entirely comparable to ours, the difference being that his effect was more nearly immediate. Thompson bases his interpretation on the previous work of Kyes (9), who connected the phagocytic activity of the fixed endothelium of liver and spleen with the destruction of red blood cells and bacteria, and of Cary (10), who assigned to the same cells the rôle of antibody production. Showing that tuberculin alone causes "a profound modification of the endothelium of the

liver and spleen leading to the formation of multinucleated phagocytic giant cells," Thompson believes that this change is responsible for increased production of amboceptor.

McJunkin (11) has shown that an increase in circulating endothelial leucocytes is a feature of tuberculosis in the guinea pig, and that it may also be induced by suitable administration of non-pathogenic acid-fast bacilli. Aside from any such demonstration there is every reason to believe that the epithelioid cell so prominent in tuberculous lesions is closely related to the cells which the authors just mentioned are concerned with.

Accepting and assigning full value to such evidence as to the localization of the reaction, we feel that some measure of additional interest attaches to our experiments as showing that exaggerated antibody-producing capacity is a feature of experimental tuberculosis considered as a disease complex. Moreover, knowledge of the locus of antibody formation, granted that it were complete, would in no way satisfy our need for a better understanding of the interrelations of reactions toward different antigens.

There are very many observations showing that antibody production may be increased or diminished by the administration of substances not in themselves antigenic, and there is some evidence that the process is influenced by physiologic state and by measures which alter this. This literature was well reviewed by Hektoen (12) in 1910, and most striking results have recently been reported by Madsen (13). The immediate bearing on our problem of observations along these lines is not at present apparent.

Friedberger (14) states that in his own experiments the simultaneous administration of two different antigens diminished the response to each. It is a well known fact that guinea pigs treated with diphtheria toxin-antitoxin mixtures are more highly sensitized than are those treated with equal amounts of antitoxin or normal horse serum. In the light of our experiments it would seem possible to offer a hypothetical stimulating effect of diphtheria toxin on the production of the anaphylactic antibodies as a tentative explanation of this obscure phenomenon.

So much discussion doubtless suffices to show how very much involved is the field which must be covered in an endeavor to arrive at a just interpretation of these relatively simple experiments, and

especially in trying to develop our knowledge of the nature of the inheritance of disease resistance.

The experiments themselves agree with those of Webster (4) in showing that where we are dealing with the "capacities" which must underlie the phenomena of immunity, whether natural or acquired, the rules of specificity are no longer a sure guide to either experiment or interpretation.

With relation to the problems outlined in our introduction the experiments show definitely that the body may be much occupied with one microorganism (*i.e.* one antigenic complex) without losing its capacity to react to an unrelated one. This statement does not, of course, commit us to any view with regard to the source of the antibodies, or the nature of the stimulus evidenced by increased antibody production, particularly as to whether that stimulus is exerted by an antigenic or a non-antigenic substance.

The experiments also show that in tuberculosis that character which we have designated as allergic irritability is high with respect to an antigen not related by origin to the tubercle bacillus, and thus form the background for further study in the general direction contemplated.

SUMMARY.

The guinea pig infected with virulent tubercle bacilli develops much more anti-sheep amboceptor than do controls when given like amounts of sheep red blood corpuscles.

The curve of antibody production in the guinea pig when treated with sheep red blood corpuscles shows a departure from curves previously determined in other animals.

These facts were ascertained as part of an effort to learn more of the functional nature of the inheritable factors controlling natural resistance to disease. The nature of some of the problems involved is outlined, and the limited bearing of the experiments on these is discussed.

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THE PRODUCTION OF PERSISTENT ALOPECIA IN RABBITS BY ROENTGEN RADIATION; A STUDY OF THE MINIMUM DOSE REQUIRED AND THE CONSISTENCY OF THE REACTION.

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(Received for publication, June 2, 1924.)

Biological reactions to Roentgen rays exhibit, in general, fairly wide variations even under conditions which are assumed to remain constant. It is not easy in most cases to decide how much of the inconsistency is the result of inexact diagnosis, how much is caused by errors in dosage, and how much is referable to normal biological variation. Many commercial Roentgen ray plants function more or less erratically; and the complexities of Roentgen radiation, and of its behavior, render difficult, and in some cases impossible perhaps, the exact prediction or the measurement of dosage.

Skin reactions offer obvious advantages for experimental study of Roentgen ray effects since they may be followed in the living animal over long periods, and in particular, the alopecia reaction commends itself because it is so readily recognized. The authors are making a series of experiments on small animals in order to determine, if possible, the degree of normal biological variation to be expected in this particular reaction, under minimum radiation, after uncertainties of dosage have been eliminated in so far as is possible. This paper presents in some detail the study of a single series of fourteen rabbits treated on the abdomen with soft rays—the only series which has been under observation for a length of time sufficient to justify a statement of the results. The animals were carefully selected for reasonable uniformity of weight, absence of pigmentation on the abdomen, general health, and particularly for freedom from nose disease.

The Source of Radiation.

A standard broad-focus Coolidge tube is operated on 60 cycle current, the inverse part of which is suppressed by two kenetrons—one at either terminal of the transformer secondary. The current is kept constant by a modified Victor-Kearsley stabilizer. When the machine is in use, both the stabilizer control and the fine adjustment of the field current of the primary current generator are constantly under control of the operator. The milliammeter is connected between the halves of the transformer secondary, at which point the circuit is grounded.

The tube occupies the upper compartment of a large lead-lined cabinet, the lower compartment of which is fitted with an adjustable shelf, for supporting the board on which the animal is placed. The lead-covered partition, between the compartments, has an aperture under the tube, and is fitted with a filter slide and a lead shutter. A narrow vertical slot in the back of the cabinet permits the ionization chamber to be inserted at any desired height. All parts of the cabinet are ventilated by means of an external fan, which forces air through lead-protected channels.

The constancy of operation of this plant was checked by means of ionization measurements, made with a standard guard-plate chamber, during a period of more than a year before animal experiments were started. No variation in efficiency amounting to more than 2 per cent was found. During this time, the outfit was operated only for the purpose of testing. It is to be expected, of course, that, with prolonged use, the tube and the kenetrons will undergo slight changes. The tube becomes coated with tungsten vapor, which absorbs the soft rays to some extent. The tests proved only that the plant does not behave erratically. Measurements of the efficiency of the tube as a function of direction disclosed no lack of uniformity over the small angular field used in the present experiments.

In the experiments described in this paper, the tube was operated at 30 kilovolts (peak) and 22 milliamperes, at a target distance of about 27 cm. Much care was exercised to keep the target distance constant throughout the experiments. The care with which the animals were adjusted to proper position, as well as the slight eccentricity of those

parts of the radiation field which are of importance in the final result, makes it reasonable to suppose that the actual distance did not differ in any case from the mean value by more than about 1 per cent. The radiation was very nearly normal to the skin in all cases. The rays were filtered at a target distance of about 20 cm. through thin bristol board covered on one side with aluminium leaf for electrostatic protection.

Preparation and Exposure of the Animals.

The treatments were given through apertures in a shield of lead-filled sheet rubber. This shield, 40 cm. square, is 1.5 mm. thick except in the middle, where a rectangular portion 15 by 18 cm. is built up to a thickness of 3 mm. In this thick part are seven apertures—two parallel rows of three circular holes, each 1 cm. in diameter, and one elongated aperture at one end of the double row, for purposes of identification. All of the apertures lie within a rectangle 3 by 6 cm. The shield is prepared for use by coating the middle part on the under side with adhesive mixture, taken from surgeon's tape by applying the adhesive surface of the tape to the shield, dampening with xylol, and stripping off the fabric.

The animal, with abdomen shaved and washed, is placed on a board, and sandbags are arranged on either side to restrict its movements. The shield is then put in place, and the adhesive surface pressed into good contact with the skin. A frame of wires supports the free parts of the shield. With this arrangement, only a small part of the weight of the shield rests on the animal, and the apertures are maintained in a fixed relation to the skin during treatment. When the shield has been put in place, a lead-covered board, with aperture 12 by 15 cm., is placed over all, and supported at a fixed height above the first board on four pillars. The animal is adjusted vertically, until the surface of the abdomen is exactly flush with the lower surface of this board, in order to define the target distance.

Before treatment, each of the apertures of the shield save one, the large one, is covered with a little piece of leaded rubber. The lengths of exposure of the various areas are controlled by removing these patches, in a definite order, at proper intervals. After the treatment, the adhesive is removed from the skin with ether and alcohol.

Ionization Measurements.

Ionization measurements were made at intervals with apparatus previously described.[1] The small chamber (about 5 mm. in diameter), half imbedded in the surface of a wax phantom, which is used in place of the animals, is carefully adjusted to the skin-target distance. Two pieces of sheet lead laid on the wax, one on either side of the chamber, with their straight edges parallel to the chamber and equidistant from it, serve to define the width of the exposed portion of wax. In measurements made to follow up possible gradual changes in tube efficiency, an opening of 1.25 cm. was used.

In order to evaluate animal dosage in terms of ionization, it is necessary to estimate the variation of dose with aperture size caused by scattering. For this purpose, measurements were made with various widths of aperture between the lead plates. When the aperture was increased from 1.25 cm. to 3.75 cm., the ionization rate increased by 3 per cent. It appears, therefore, that measurements made with the smaller aperture should be decreased by about 1.5 per cent to correspond with the small apertures used with the animals. The proximity of the various rayed areas of the animal should introduce a slight scattering error. A consideration of all of the factors involved indicates, however, that it is negligible in the cases which have a bearing on the results of these experiments.

RESULTS.

Chart 1 contains the records of all of the animals treated, with the exception of one, No. 37, which developed multiple abscesses in the treated area after the 3rd week. There is no reason to suppose that these abscesses were caused by radiation, since they occur frequently in rabbits otherwise normal.

The black circles represent alopecia persisting at least 10 weeks after treatment. The white circles represent a definite regrowth of hair after an interval indicated, in weeks, by the numbers within.

Rabbits 1 to 8 (Group A) were rayed consecutively; that is, without allowing the plant to be used for other purposes. Likewise, the

[1] Clark, H., *Am. J. Roentgenol. and Radium Therap.*, 1924, xi, 445.

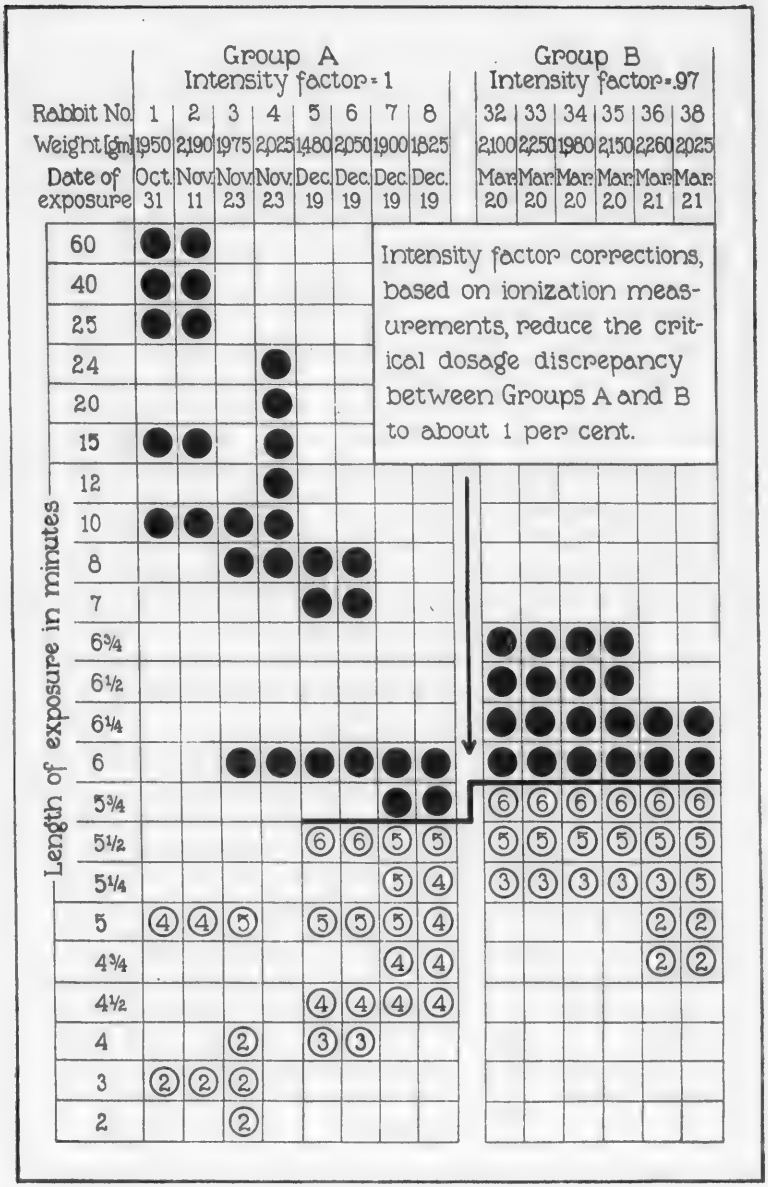
Group A
Intensity factor = 1
Group B
Intensity factor = .97
Rabbit No. 1 2 3 4 5 6 7 8 32 33 34 35 36 38
Weight [gm] 1950 2190 1975 2025 1480 2050 1900 1825 2100 2250 1980 2150 2260 2025
Date of exposure Oct. 31 Nov. 11 Nov. 23 Nov. 23 Dec. 19 Dec. 19 Dec. 19 Dec. 19 Mar. 20 Mar. 20 Mar. 20 Mar. 20 Mar. 21 Mar. 21
Length of exposure in minutes
60
40
25
24
20
15
12
10
8
7
6¾
6½
6¼
6
5¾
5½
5¼
5
4¾
4½
4
3
2
Intensity factor corrections, based on ionization measurements, reduce the critical dosage discrepancy between Groups A and B to about 1 per cent.

CHART 1.

animals of Group B were rayed consecutively. In the interval between the treatments of the two groups, the machine was almost constantly in use, and the ionization measurements indicated a consequent decrease of 3 per cent in the soft ray efficiency. The necessary correction is embodied in the intensity factors shown at the top of the chart.

When these factors are taken into account, the discrepancy in critical dosage between the groups, amounting to about 4 per cent on the exposure scale, is reduced to about 1 per cent. The experiments disclose, therefore, no evidence of any biological variation whatsoever. The weighted mean value of exposures, corresponding to all circles contiguous to the critical dose line on the chart, corresponds, for unit intensity factor, to an exposure of 340 seconds. The spacing of the exposures is such that this mean value may differ from the true critical exposure by about 2 per cent, which is just within the limits of accuracy assumed for the dosage.

This mean value corresponds, according to the ionization measurements, to the production of about 2.04×10^{15} ions (of either sign) per gm. of tissue. The correspondence entails, however, an additional chamber calibration error of perhaps 2 per cent, and it rests on the assumption that the method of phantom measurement is valid—an assumption not entirely free from objection.

In experiments of this kind, it is difficult to exclude unconscious personal bias in making diagnoses. In view of this fact, the work has been divided sharply into two parts. All treatments and ionization measurements were made by one of us (Clark) while all diagnoses were made by the other (Sturm, kindly assisted by Dr. James B. Murphy) without knowledge of the measurements.

This paper is presented as a record of the experiment without any conclusions as to the general validity of the results. Whether, in other circumstances, the reaction would have been less consistent, and whether, in the present ones, a larger number of animals would exhibit greater variations, are questions on which no opinion is ventured.

SUMMARY.

Seven areas on the abdomen of each of fourteen rabbits were exposed to soft Roentgen radiation of constant quality in doses

varying, in the region of the value critical for the production of persistent alopecia, by regular steps of about 4 per cent. Without exception the critical dose was found to lie between two such values, the upper limit being represented by exposures on eight of the animals and the lower on ten of them. With certain reservations, the critical dose corresponds to the production of 2.04×10^{15} ions of either sign per gm. of tissue.

ATTEMPTS TO TRANSMIT RHEUMATIC FEVER TO RABBITS AND GUINEA PIGS.

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INTRODUCTION.

The reticence of investigators to report their failures has probably retarded the solution of many problems. Much labor might be spared if a new worker in a subject had at his disposal a detailed record of the unsuccessful as well as the successful endeavors of his predecessors, a record so complete that he could avoid the repetition of details in technique that impress him as being possible causes of previous failures. It is such a record that we desire to report. In doing so, it is fully appreciated that unsuccessful transmission experiments never prove conclusively the non-existence of a virus in the inocula employed.

The search for the etiological agent of rheumatic fever has occupied the attention of many investigators. Of the several microorganisms ascribed to this rôle the *Streptococcus viridans* has taken the foremost position. But a number of unexplained objections have restrained the majority of workers from accepting this bacterium as the cause of the disease. Perhaps the most important objection is the failure of laboratory animals inoculated with the *Streptococcus viridans* to develop the characteristic pathological lesion of rheumatic myocarditis, the so called submiliary nodule, or Aschoff body.

The production of experimental rheumatic myocarditis by the intravenous inoculation of rabbits with *Streptococcus viridans* was first attempted by Bracht and Wächter (1). They described small foci of parenchymatous necrosis which became surrounded by lymphocytes, polymorphonuclear leucocytes, and fibroblasts; and which tended to heal with scar formation. These lesions, now quite generally known as the Bracht-Wächter bodies, did not resemble Aschoff bodies, but rather the focal embolic myocarditis of subacute bacterial endocarditis.

Thalhimer and Rothschild (2) and Cecil (3) found no Aschoff bodies in the hearts of rabbits inoculated with *Streptococcus viridans*, but only lesions similar to those of Bracht and Wächter.

Jackson (4), Coombs, Miller, and Kettle (5), and Rosenow and Coombs (6) all report the production in rabbits injected with *Streptococcus viridans* of myocardial lesions closely resembling Aschoff bodies. Their descriptions and illustrations, however, are not convincing. The chief points of dissimilarity are the necrotic changes, the presence of streptococci, often in considerable numbers, the tendency to radiating configuration, and the absence of the characteristic large, multinucleated type cell of the Aschoff body. The myocarditis described by Herry (7) in rabbits injected with "*endotoxine rhumatismale*" prepared from streptococci will be discussed later.

Besides these studies by the adherents of the streptococcal etiology of rheumatic fever there have been two investigations based on the hypothesis that the disease is caused by a filterable virus. De Vecchi (8), in 1912, reported the production of a myocarditis, which he regarded as very closely approximating that of rheumatic fever, by the inoculation into rabbits, dogs, rats, and mice of bacteria-free blood from patients with rheumatic fever. At the time of inoculation his animals were also injected with repeated small doses of a 1:5,000 solution of epinephrine. This latter procedure was intended to increase the susceptibility of the heart muscle to the action of the virus. Of the several species employed the rabbits showed the most marked myocardial lesions. Uninoculated control rabbits and those injected only with epinephrine sometimes showed lesions but not so extensive as those receiving serum from patients with rheumatic fever. No control experiments with epinephrine and normal human serum were made. Further reference to this work will be made in the discussion. More recently Natali (9), using only rabbits, has repeated de Vecchi's observations.

EXPERIMENTAL.

We have attempted to transmit rheumatic fever to rabbits and guinea pigs by injecting them with various materials obtained from patients suffering with this disease. The materials selected were whole blood, serum, joint fluid, pleural fluid, throat washings, and tonsil tissue. Thus were included those body fluids easily available, which might rationally be assumed to contain the virus of the disease during its acute stage. The frequent association of tonsillitis with rheumatic fever explains the use of throat washings and of tonsil tissue.

Practically all of these substances were obtained either before the patients had received antirheumatic medication, or after a sufficient time following the discontinuance of the treatment for its effect

to have disappeared. The clinical data on the patients are given in Table I. In each protocol the relation to such medication has been noted.

Methods.

The following inocula were used.

1. Blood.—The blood was withdrawn by venipuncture during the acute febrile state while the polyarthritis was still developing. In some instances serum was used, but in most the whole uncoagulated blood, usually drawn into a syringe which had been previously washed with a 10 per cent solution of sodium citrate. In one experiment a 3 day blood culture in broth was used.

2. Joint Fluid.—The fluid was aspirated from acutely inflamed joints, sometimes within a few hours after the onset of symptoms in the joint punctured. The earliest possible exudate was thus obtained. Anaerobic cultures of joint fluid made according to various modifications of the Smith-Noguchi method were also used for inoculation. Such cultures were always free from any demonstrable bacteria.

3. Pleural Fluid.—This was withdrawn during an acute pleurisy with effusion in a patient with rheumatic fever. Culture of this fluid showed no growth.

4. Throat Washings.—The patient's throat was rinsed with about 15 cc. of Ringer's solution. The washings were then filtered through a Berkefeld N filter, with suction from the house vacuum system. Cultures of the filtrates were always sterile.

5. Tonsillar Material.—The tonsils were removed from patients under gas-ether-chloroform anesthesia. Within 1 hour the tonsils were minced until reduced to a pulp which was suspended in sterile Ringer's solution and centrifuged at slow speed for a few minutes to remove the coarser particles. Some inoculations were made with the suspension in this state and some with the filtrate passed through a Berkefeld N candle. The filtrates were invariably sterile in ordinary media.

Transfer Inoculations.—The materials used for transfer inoculation were blood and a suspension of heart muscle and spleen. These suspensions were made by mincing the tissues, mixing the pulp in sterile Ringer's solution, and centrifuging a few minutes at slow speed to throw down the coarser particles. In one instance anaerobic Smith-Noguchi cultures (ascitic fluid-kidney tissue) of joint fluid of Rabbit H-63 (Series III) were used. The same general plan was followed for transfer injections as in the initial inoculation.

Animals.—The animals employed were young, healthy guinea pigs and rabbits. The guinea pigs were always inoculated intraperitoneally, the rabbits intravenously into the ear veins with blood and joint fluid, but intraperitoneally with all other inoculants. This was necessary because it was found that the intravenous injection of such materials as tonsil suspension invariably proved fatal within a few hours.

Subcutaneous inoculations were also made into the abdominal wall of both rabbits and guinea pigs in order that any local reaction might be more easily observed and also with the hope that subcutaneous rheumatic nodules might be produced. A few intraarticular inoculations were made into the knee joints of rabbits.

Clinical Observations on the Animals.—The animals were fed on a diet of hay, oats, bread, greens, and water. They were weighed daily. Their rectal temperatures were taken twice a day. It was early found necessary to sterilize the thermometers in lysol solution and alcohol to prevent the spread of any epidemic gastrointestinal disease.

The animals were examined daily for evidence of arthritis, subcutaneous nodules, diarrhea, snuffles, or any other signs of illness. X-rays were made of any suspicious joints in order to avoid mistaking a bone fracture for joint involvement.

Routine Autopsy Procedure.—The animals were killed by a blow on the occiput. The chest was opened asceptically and blood collected into a large capillary pipette from the heart or great vessels. This blood was cultured routinely in dextrose agar plates and deep tubes, and sometimes also in broth and used for the inoculation of other animals as indicated in the tables. If inspection of the viscera suggested the possibility of pneumonia, pleuritis, pericarditis, or peritonitis, film preparations and cultures were made. If heart and spleen were to be used for the transfer inoculations a piece of each was removed and placed in a sterile dish to be treated as described elsewhere. Pieces of the following organs were fixed for histological examination: heart, lung, spleen, liver, and kidney.

The skin of the animal was then removed and the skeletal musculature and joints dissected and carefully examined. Joints showing such abnormalities as increased fluid content, congestion, or swelling of the periarticular tissues were cultured and then fixed for future histological examination.

Histological Examination.—Tissues to be examined microscopically were fixed in either Zenker or Müller-formol, and a piece of the heart muscle was always fixed in absolute alcohol as well. Sections were cut in paraffin and stained with eosin-methylene blue or with hematoxylin and eosin (depending on the fixation). Sections of the heart were stained also with the Unna-Pappenheim methyl green-pyronine stain.

Controls.—Control animals, both rabbits and guinea pigs, were inoculated with normal human serum and whole blood, joint fluid from a case of arthritis deformans, control culture media, normal rabbit blood, and suspension of normal rabbit heart and spleen. These animals as well as uninoculated ones were kept under identical conditions and were autopsied and examined with the same care.

RESULTS.

Twenty-seven rabbits and fourteen guinea pigs were inoculated according to the methods described with body fluids and tissues of

patients suffering from rheumatic fever. Transfer inoculations, *i.e.* rabbit to rabbit or guinea pig to guinea pig, brought the total number of animals used in the experiments to 67 rabbits and 40 guinea pigs. These transfer inoculations were systematically carried out in a number of series in spite of the absence of symptoms of disease, because such negative evidence does not prove the non-existence of a virus in the body of the animal inoculated. It is well known that several passages may be necessary to adapt a virus to a new environment, and to render it parasitic and pathogenic for its new host species.

A definite arthritis occurred in only two animals, Rabbit H-63 (Series III) and Guinea Pig B-86 (Series xiii).

Rabbit H-63 had received the blood and suspension of heart muscle from a rabbit inoculated with whole blood from a patient with rheumatic fever. This rabbit had a mild fever, 104–104.5°, for a number of days. 26 days after inoculation the left ankle became swollen and hot. An x-ray picture showed the bony structures to be uninjured. On the 28th day the animal was sacrificed. The periarticular tissues were congested and edematous. Smears of this edema fluid showed mononuclear and polymorphonuclear leucocytes, but no bacteria. The joint cavity contained an increased amount of fluid, smears of which showed no cells and no bacteria. Cultures of the joint fluid and of the periarticular tissue juice in ordinary media and in Smith-Noguchi kidney-ascitic fluid showed no growth.

Efforts to repeat this occurrence by the inoculation into other rabbits of the blood of Rabbit H-63 and of the anaerobic cultures of the joint fluid were unsuccessful.

Guinea Pig B-86 (Series xiii) was injected intraperitoneally with 3 cc. of filtered throat washings from a patient with rheumatic fever during a relapse accompanied by severe pharyngitis. Cultures of the filtrate in blood agar showed no growth. The animal had fever (103°) from the 3rd to 7th days after inoculation and again for 2 days before death. It developed swelling of the right ankle on the 15th day without other evidence of disease and was sacrificed the following day. A complete autopsy revealed no pathological changes except swelling about the left ankle and an excess of fluid in the joint. No evidence of trauma was found. Anaerobic culture of the joint fluid in ascitic broth was sterile. Cultures of heart muscle and kidney were sterile. Two out of four cultures of heart's blood were contaminated by staphylococci; the others showed no growth. Transfer inoculations from this animal were negative.

Although these observations were unique in our entire series of experiments they are of considerable interest. Non-bacterial arth-

TAB

Sources of Material and Occurrence of Myo

Patients furnishing material.				Material used
Case No.	Age. Sex. Hospital. No.	No. of previous attacks of rheumatic fever.	Clinical features.	Material.[1]
I	13 yrs. M. 4269	0 (4)[3]	Fever; slight arthritis disappearing spontaneously; moderately severe pancarditis. Mitral stenosis and regurgitation; subcutaneous rheumatic nodules. Six blood cultures negative. One attack of heart failure relieved by digitalis. At the end of 4 mos. he left the hospital against advice. Died of heart failure 7 wks. later. No autopsy.	Blood.
II	35 yrs. M. 4233	0	Fever; severe migratory polyarthritis; mitral endocarditis. Two blood cultures negative. Typical response to salicylate therapy. Recovery.	"
III	38 yrs. F. 4255	2	Fever; migratory polyarthritis. Severe pancarditis and pleurisy. Four blood cultures negative. Mitral regurgitation. Salicylate medication unsatisfactory because of drug idiosyncrasy; spontaneous disappearance of arthritis. Left hospital against advice. Returned 2½ mos. later in advanced heart failure and died 11 days thereafter. No autopsy.	"
IV	14 yrs. M. (Wm. E., out patient.)	2	Fever; mild polyarthritis, subcutaneous nodules. Chronic and acute myocarditis; aortic insufficiency, mitral insufficiency. Recovery. 3 yrs. later, a fourth attack in which he died. No autopsy.	Serum.[5] 3 day dextrose[4] broth culture of blood.

R. indicates rabbit; G. P., guinea pig; +, myocardial lesions found on microscopic examination (see text); −, no myocardial lesions.

[1] Unless otherwise noted, cultures, of these materials were sterile.

[2] Animal transfers are numbered so that the original inoculation is called the first generation.

[3] Attacks of chorea.

[4] Rabbit H-63. Acute non-bacterial arthritis (see text).

[5] Culture grew a *Streptococcus viridans*.

[6] No organisms demonstrable at this time; subsequently grew a *Streptococcus viridans*.

LE I.

ardial Lesions in the Inoculated Animals.

for animal inoculation.		Animals inoculated.					
Day of disease obtained.	Previous salicylate medication.	Generation.[2]					
		1st	2nd	3rd	4th	5th	6th
22nd	None.	R. −					
11th	"	" +					
		G. P. −	G. P. +				
			" " −	G. P. +			
7th	"	R. +	R.[4] +	R. +	R. −		
				" −			
		G. P. −		" +			
				G. P. +			
		" " +	G. P. +	" " −			
			" " +	" " +			
		R. +	R. +	R. +			
13th	?	" −	" +		" −		
				" −	" −		
		" −	" −	" +	" −	R. −	R. −
				G. P. +	G. P. +	" −	" −
			" −	R. −	R. −		
					" +		
			" +	" −			
				" −	" +		

TABLE

Patients furnishing material.				Material us
Case No.	Age. Sex. Hospital. No.	No. of previous attacks of rheumatic fever.	Clinical features.	Material.[1]
				Serum.[7]
				" [7]
V	36 yrs. M. 4193	3	Fever; migratory polyarthritis. Prompt response to sodium salicylate. Recovery.	Blood.
				Joint fluid, left kn (within 24 hrs. a ter onset of sym toms).
VI	12 yrs. M. 4277	0 (3)[8]	Fever; migratory polyarthritis, promptly relieved by sodium salicylate medication. Mitral regurgitation. Two relapses with fever, precordial pain, and lengthening of P-R interval in electrocardiogram. Three blood cultures negative. Tonsillectomy at end of 2nd mo. Uneventful convalescence.	Blood. Suspension of tonsi (filtered).
VII	31 yrs. F. 4312	0	Fever; severe migratory polyarthritis; severe pancarditis; tardy response to full doses of sodium salicylate. Relapse with pleurisy, marked disturbance of cardiac rhythm—auricular fibrillation and flutter. Six blood cultures negative. Prolonged convalescence.	Blood. Throat washings (f tered).
VIII	20 yrs. F. 46477 (Presbyterian Hosp.)	1	Fever; migratory polyarthritis; pericarditis with effusion; pleurisy with effusion. Mitral disease. Prolonged illness. Recovery.	Blood. Pleural fluid.

[7] Of three subsequent blood cultures, only one grew a *Streptococcus viridans*.

[8] During first relapse.

—*Continued.*

for animal inoculation.		Animals inoculated.					
		Generation.[2]					
Day of disease obtained.	Previous salicylate medication.	1st	2nd	3rd	4th	5th	6th
13th	?	R. − " + G. P. ? " " −	R. − " − G. P. + " " − " " +				
23rd	?	R. + " +					
25th	?	" −					
18th	None.	" −	R. − " + " − " +				
19th	"	" − " + " −					
21st[3]	" for 10 days.	" +	" −				
66th	" for 8 wks.	G. P. +					
4th	"	R. −	" +				
6th	"	G. P. +	G. P. −				
14th	?	R. − " +	R. −				
17th	?	G. P. +					

TABLE I

Patients furnishing material.				Material used
Case No.	Age. Sex. Hospital. No.	No. of previous attacks of rheumatic fever.	Clinical features.	Material.[1]
IX	23 yrs. M. 4313	0	Fever; migratory polyarthritis; mitral regurgitation; lengthening of P-R interval in electrocardiogram; improvement under sodium salicylate therapy. Seven blood cultures negative. Tonsillectomy. Recovery.	Suspension of tonsils.[9] Suspension of tonsils (filtered). 18 hr. serum broth culture of tonsil suspension.[10] 18 hr. serum broth culture (filtered). 18 hr. serum broth culture (filtered) plus 1.0 cc. broth culture *Streptococcus viridans*.
X	14 yrs. M. 4348	0	Fever; mild polyarthritis which disappeared without sodium salicylate medication; mitral and aortic regurgitations. Tonsillectomy followed by relapse. Five blood cultures negative. Slow convalescence. Death from heart failure 5 mos. after discharge. No autopsy.	Suspension of tonsils.[11] Suspension of tonsils (filtered).
XI	17 yrs. F. 4126	1	Repeated bouts of fever; mild transitory polyarthritis. Evidence of pancarditis. Three blood cultures negative. Tonsillectomy in 11th mo. Prolonged illness; slow convalescence. Two subsequent admissions. Death. *Autopsy.*—Numerous typical Aschoff bodies in myocardium; chronic and acute mitral and aortic endocarditis, and acute verrucous tricuspid endocarditis.	Suspension of tonsils (filtered).
XII	29 yrs. F. 4111	0	Fever; migratory polyarthritis, lengthening of P-R interval in electrocardiogram. Mitral and aortic valvular disease. Typical response to sodium salicylate. Two blood cultures negative. During convalescence an acute pharyngitis. Tonsillectomy. Recovery.	Throat washings.[12] Suspension of tonsils (filtered).

[9] Cultures grew hemolytic and non-hemolytic streptococci and Gram-negative diplococci.
[10] Gram-positive and Gram-negative cocci and bacilli.
[11] Cultures grew *Staphylococcus albus* and non-hemolytic streptococci.
[12] Cultures grew *Streptococcus viridans*, indifferent streptococci, and diphtheroids.

—*Continued.*

for animal inoculation.		Animals inoculated.					
		Generation.[2]					
Day of disease obtained.	Previous salicylate medication.	1st	2nd	3rd	4th	5th	6th
51st	3 gm. sodium salicylate a day for several wks.	R. −					
51st	3 gm. sodium salicylate a day for several wks.	" −					
51st	3 gm. sodium salicylate a day for several wks.	" −					
51st	3 gm. sodium salicylate a day for several wks.	" +					
51st	3 gm. sodium salicylate a day for several wks.	" −					
30th	None.	G. P. −	G. P. +				
30th	"	" " +					
11th mo.	"	" " +					
25th	Sodium salicylate discontinued 1 mo. before.	R. +					
25th	Discontinued 2 mos. before.	G. P. −					
		" " +					

TABLE I

Patients furnishing material.				Material used
Case No.	Age. Sex. Hospital. No.	No. of previous attacks of rheumatic fever.	Clinical features.	Material.[1]
XIII	19 yrs. F. 46033 (Presbyterian Hosp.)	0	Fever; migratory polyarthritis. Improvement with subsequent relapse accompanied by severe pharyngitis. Mitral disease (?). Recovery.	Throat washings (filtered).
XIV	34 yrs. M. 4350	0	Fever; migratory polyarthritis; mitral endocarditis(?). Improvement under sodium salicylate therapy. Tonsillectomy followed immediately by relapse. Improvement under sodium salicylate therapy. Severe relapse with pericarditis and pleurisy, with effusion. Seven blood cultures negative. Recovery.	Suspension of tonsils (filtered). Joint fluid, left ankle.
XV	18 yrs. M. 4367	2	Fever; severe migratory polyarthritis; mitral and aortic valvular disease. Relief of symptoms by sodium salicylate followed by relapse after discontinuance. Joint aspirated at this time. Three subsequent relapses. Prolonged convalescence. Recovery.	Joint fluids, both knees. (Aspirated within a few days after onset of symptoms.)
XVI	19 yrs. F. 4329	0	Fever; migratory polyarthritis; pancarditis; pleurisy. Subcutaneous rheumatic nodules. Four blood cultures negative. Tonsillectomy. Slow convalescence.	Smith-Noguchi cultures of joint fluid left knee. Right knee.

[13] Guinea Pig B-86. Acute non-bacterial arthritis (see text).

ritis does not occur spontaneously in healthy rabbits or guinea pigs. The animals were in a good state of nutrition and had been gaining weight steadily until 2 or 3 days before the appearance of joint involvement. Trauma cannot be definitely ruled out, even though x-ray gave no evidence of injury to the hard parts.

A fairly common finding in both the rabbits and guinea pigs was the presence of bright red petechial hemorrhages into the synovial

Concluded.

Animal inoculation.		Animals inoculated.					
Day disease gained.	Previous salicylate medication.	Generation.[2] 1st	2nd	3rd	4th	5th	6th
40th	Sodium salicylate 4 gm. a day for 3 wks.	R. +	R. −	R. −	R. −		
			" −				
		G. P. +[13]	G. P. −				
			" " +				
31st	Sodium salicylate daily for 18 days. 4 gm. the day before tonsillectomy.	" " −	" " +				
		" " −					
36th	None for 6 days.	" " +	" " −				
17th	" " 5 "	" " +	" " +				
23rd	"	" " +					
		" " −					
30th	"	" " +					
		" " +					
		" " −					

membranes of the joints and into the periarticular tissues. As they were found in uninoculated control animals, and as microscopic examination showed them to be fresh and devoid of any inflammatory reaction, they were thought to be agonal in origin.

Myocardial Lesions.—The microscopic examination of the hearts of all of the rabbits and guinea pigs revealed the presence of myocardial lesions in a large percentage. Their presence or absence has been indicated by + and − marks in Table I.

The lesions most commonly consisted of groups of lymphocytes to which were sometimes added endothelial leucocytes in varying proportions. Polymorphonuclear eosinophils and more or less mature connective tissue cells were not uncommon. Plasma cells, mast cells, and polymorphonuclear neutrophils were rather rare. The

TABLE II.

The Relation of Age to the Incidence of the Myocardial Lesions. (Weight Has Been Used as an Approximate Index of Age.)

	Rabbits.			
	Less than 500 gm.	500–999 gm.	1,000–1,500 gm.	Over 1,500 gm.
No. of rabbits receiving initial inoculations	2	14	9	2
" " such rabbits showing myocardial lesions	1	3 (21%)	6 (66%)	2
No. of rabbits receiving transfer inoculations	5	26	7	1
" " such rabbits showing myocardial lesions	1 (20%)	9 (29%)	4 (57%)	1
Per cent of the total in each weight group showing myocardial lesions	28	30	62	

	Guinea pigs.		
	Less than 300 gm.	300–500 gm.	Over 500 gm.
No. of guinea pigs receiving initial inoculations	9	10	2
" " such guinea pigs showing myocardial lesions	6 (75%)	6 (60%)	2
No. of guinea pigs receiving transfer inoculations	10	5	4
" " such guinea pigs showing myocardial lesions	7 (70%)	4 (80%)	2
Per cent of the total in each weight group showing myocardial lesions	68	66	

arrangement of the cells was not strikingly constant. The various types of cells seemed to be mixed together without order. The lesions tended to assume an elongated, irregularly elliptical shape, their long diameters parallel to the adjacent muscle fibers. The more extensive lesions filled the spaces between a number of fibers. In

such instances the muscle cells sometimes appeared normal and sometimes showed loss of striation. But evidences of necrosis were uniformly absent except in the hearts of rabbits which had had a terminal bacteremia.

The lesions were most frequently found in the myocardium of the papillary muscles and septum, less often in the walls of the ventricles and only occasionally in the auricles. Some lesions occurred subendocardially and subepicardially. None was ever found in the valves.

In the rabbits the incidence of the myocardial lesions was roughly proportional to their ages, but this was not true for the guinea pigs (see Table II). As the exact ages of the animals were not known,

TABLE III.

Incidence of Myocardial Lesions in Animals Injected with Human and with Homologous Substances.

Of 27 rabbits inoculated with human material	12 (or 44 per cent) showed heart lesions.
Of 40 rabbits inoculated with transfer material from other rabbits	19 (" 47 " ") " " "
Of 21 guinea pigs inoculated with human material	14 (" 66 " ") " " "
Of 19 guinea pigs inoculated with transfer material from other guinea pigs	13 (" 68 " ") " " "

their weights were used as approximate indexes of age. In these tables the animals have been separated into two groups, those which had been injected with human material (initial inoculation) and those which had been injected with transfer material from other animals of their own species (transfer inoculations).

This grouping is necessitated by the suspicion which naturally comes to mind that the lesions might have been the result of inoculations with foreign proteins and be similar in nature to those described by Longcope (10). That this is not the case is shown by Table III.

The lesions occurred with equal frequency in animals receiving human material and in those receiving blood and tissue suspensions from animals of the same species, and cannot, therefore, be regarded as a response to the inoculation of foreign proteins.

Lesions in every way identical with those described were found in control animals, both inoculated and uninoculated.

DISCUSSION.

With the exception of the two instances of outspoken arthritis already discussed no evidence of successful transmission of rheumatic fever was encountered in these experiments. As has been mentioned, non-bacterial arthritis is not met with in healthy rabbits and guinea pigs, and its occurrence in animals injected with material from patients with rheumatic fever is interesting and suggestive. But the presence of arthritis in those two animals does not justify the conclusion that they were infected with rheumatic fever.

The transmission of rheumatic fever to laboratory animals is an important problem, one which must not be abandoned, for on its solution may rest the elucidation of the etiology of the disease. Our failure to accomplish it does not convince us that the active agent was not present in some of the materials with which we were working. A more reasonable explanation for our failure seems to be the inability of the agent to gain a foothold in the species of animal employed under the conditions of our experiments. And it is with the hope that other workers may be hereby stimulated to attack the problem that this report is published.

The microscopic lesions in the hearts of the inoculated animals are of particular interest for several reasons. They might have been mistaken for Aschoff bodies, for occasionally one was seen which on casual observation could have been passed as an Aschoff body, not typical, to be sure. But one might not expect the tissues of the rabbit or guinea pig to react exactly as human tissues do to a human virus.

As lesions apparently identical were found in uninoculated control animals, one is forced to conclude that they occur spontaneously in rabbits and guinea pigs. This point is dealt with in more detail in other communications (11). It is obvious, therefore, that when myocardial lesions are discovered in animals subjected to experimental inoculation great caution must be exercised in the interpretation of their significance. As a similar myocarditis was considered

by de Vecchi and Natali to be proof of the production of experimental rheumatism in their rabbits, and comparable foci were ascribed by Herry to the action of an "*endotoxine rhumatismale*" from streptococci, we feel that the interpretation of these authors of the etiology and nature of the lesions found by them cannot be accepted without further proof.

SUMMARY.

In a series of attempts to transmit a virus from patients in the acute stages of rheumatic fever, twenty-seven rabbits and fourteen guinea pigs were inoculated with one of the following materials: whole blood, serum, joint fluid, pleural fluid, throat washings, suspensions of tonsil tissue. Subsequent transfer inoculations from animal to animal brought the total number of animals employed in the experiments to 67 rabbits and 40 guinea pigs. Only two animals developed an acute non-bacterial arthritis. No other evidence of successful transmission of the disease was obtained.

In about one-half of the rabbits and two-thirds of the guinea pigs myocardial lesions were encountered which consisted of interstitial accumulations of lymphocytes and endothelial cells. Similar lesions were found in control animals.

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SPONTANEOUS INTERSTITIAL MYOCARDITIS IN RABBITS.

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PLATES 20 AND 21.

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During a series of experiments in which attempts were made to transmit to rabbits and guinea pigs a virus assumed to be present in the body fluids of patients suffering with rheumatic fever, certain myocardial lesions were encountered in the inoculated animals. The results of these inoculation experiments have been reported in the preceding communication (1). Although the experiments failed to demonstrate a virus in the materials employed, they brought to light the fact that in the hearts of uninoculated rabbits kept as controls, lesions were present similar to those found in the animals subjected to inoculation. This led to a systematic investigation of the hearts of apparently healthy stock rabbits. The myocardial lesions found in these animals constitute the subject of the present paper.[1]

Despite its obvious importance for experimenters with animals no description of spontaneous myocarditis in rabbits could be found in literature. Bell and Hartzell (2) in a paper on spontaneous nephritis in rabbits state that while they did not study the hearts of their nephritic rabbits systematically, they found in three hearts of which many sections were cut "foci of lymphocytes similar to those described by Longcope, and attributed by him to the action of foreign protein." Wright and Craighead (3) in a study of infectious motor paralysis in young rabbits mention that sections of the myocardium of two animals were examined and "a few focal lesions found." In neither instance was the condition given more than the brief mention quoted.

A few spontaneous diseases of rabbits have attracted considerable attention. Spontaneous arterial degeneration has been described by Miles and Johnstone (4), and by Pearce (5); spontaneous nephritis by Ophüls (6), Bell and Hartzell (2),

[1] A preliminary report of this study has recently appeared (Miller, C. P., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 521).

and Twort and Archer (7); spontaneous encephalitis by Wright and Craighead (3), McCartney (8), Oliver (9), Levaditi, Nicolau, and Schoen (10), Goodpasture (11), and others.

Material and Methods.

This report is based on the study of the hearts of thirty-four apparently healthy adult rabbits. No animals have been included in this series which, while under observation, manifested any clinical evidences of disease—snuffles, ear canker, diarrhea, cutaneous or subcutaneous infections. It is interesting to note in passing, however, that myocardial lesions identical with those described below occurred in rabbits suffering from snuffles and from rabbit septicemia, but not with appreciably greater frequency than in the animals which constitute the series here reported.

The observations extend over a period of $3\frac{1}{2}$ years and the animals employed were obtained from a variety of sources. The myocardial lesions encountered cannot, therefore, be regarded as peculiar to a special strain of rabbits.

Most of the rabbits were kept under observation for periods ranging from a few days to 2 months. They were weighed twice a week and were periodically examined for clinical evidences of disease. When sacrificed the animals were killed by a blow on the back of the head. A complete autopsy was performed, but only the hearts were prepared for microscopical examination. They were washed in Ringer's solution, examined macroscopically, and fixed in Zenker's fluid. Prior to imbedding, the hearts were cut up into segments which were diagramed in order to facilitate the orientation of the sections. Sections were cut in paraffin and stained with eosin and methylene blue as routine or with Giemsa's stain. Several sections were cut from each block,—so that the walls of all four chambers and usually all of the valves were examined.

RESULTS.

Table I gives the following data about each rabbit: length of the observation period, maximum weight, gross pathological changes found at autopsy, and the essential facts concerning the microscopical appearance of the myocardial lesions, their number, size, and predominating cells.

The longest period of observation was 124 days; and for half of the rabbits it was longer than a week. At the time of autopsy the

animals weighed 400 to 3,200 gm.—the majority between 1 and 2 kilos. The weights have been used as a rough index of the age of the animals as their exact ages were not known. The macroscopic appearance of the viscera was normal in twenty-five of the thirty-four rabbits. Eight, or 23 per cent, suffered from coccidiosis of the liver, but this condition was not regularly associated with the myocarditis. Myocardial lesions were found in twenty, or two-thirds of the animals. They occurred less frequently in the younger animals, and were also somewhat less common in the years 1923 and 1924, when the general condition of the stock rabbits had improved; that is to say, when their general nutrition was better and they suffered less from snuffles and coccidiosis than those examined in previous years.

Description of the Myocardial Lesions.

Of the thirty-four rabbits, twenty, or 60 per cent, showed myocardial lesions. In some hearts they were numerous enough to be found in every section, while in others search through a number of sections was necessary to demonstrate their presence. The lesions were never visible to the naked eye. Upon microscopical examination they were found to be distributed as follows, in order of frequency: papillary muscles, septum, left ventricle wall, right ventricle wall, auricles. No lesion was ever found in the valves or at the bases of valves. Some occurred immediately beneath the epicardium and endocardium (Figs. 4 and 5), but the majority were within the myocardium itself. Most frequently, they lay in the interstitial connective tissue framework, occasionally close to a medium size blood vessel (Figs. 7 and 8).

Microscopically the lesions appeared as collections of cells more or less densely packed, lying between the muscle fibers. In size they ranged from small groups of six to ten cells up to areas the size of a high power field. They were usually elliptical with their long axes parallel to the muscle fibers. Some were diffuse, appearing as cords of cells filling the spaces between a number of muscle fibers.

For convenience in description the lesions may be arbitrarily divided into two groups according to the predominating cell type. Those in the first group were most frequently encountered. They consisted almost entirely of cells with irregularly round nuclei and very little

TABLE I.

Myocardial Lesions in Apparently Healthy Rabbits.

Animal No.	Period of observation.	Maximum weight.	Autopsy findings.	Myocardial lesions.		
				Number.	Size.	Predominant cell.
	days	*gm.*				
H-3	86	1,650	Negative.	Several.	Medium.	Lymphocytes; endothelial leucocytes.
H-27		700	"	Few.	Small.	"
H-71	77	1,400	"	Several.	" and medium.	" endothelial leucocytes.
H-96	80	1,420	"	" [1]	" " "	" " "
K-13	48	940	"	Few.[2]	" " "	"
K-19	6	1,400	"	"	Medium.	"
K-33	11	1,640	"	"	"	"
B-84	124	1,570	"	" [3]	Small.	"
P-19	3	1,220	"	"	"	"
P-21	2	1,500	"	"	"	" endothelial leucocytes; fibroblasts.
P-608	2	3,270	"	" [4]	"	Lymphocytes.
P-651	8	2,670	"	"	"	" endothelial leucocytes.
P-652	8	1,490	"	"	"	"
P-653	8	1,660	"	Many.[5]	" and large.	" endothelial leucocytes; fibroblasts; giant cells.
X-1	2	2,500	"	" [6]	" " "	Lymphocytes.
K-14	50	1,190	Coccidiosis.	"	"	"
P-17	1	1,300	"	Few.[7]	"	"
P-18	1	2,100	" scarred kidneys.	"	"	"
P-27	3	1,100	Coccidiosis.	"	"	" endothelial leucocytes.
H-67	53	2,180	Pregnant.	"	"	"

H-38		900	Negative.	0	0	0
H-62	53	1,840	"	0		
P-23	14	1,900	"	0		
P-24		1,300	"	0		
P-25		1,650	"	0		
P-26	3	1,270	"	0		
P-607	2	1,970	"	0		
P-629	15	2,190	"	0		
P-644	8	1,790	"	No focal lesions. Area of atrophy and fibrosis.		
X-2	2	1,870	"	0		
H-9	12	400	Coccidiosis.	0		
H-28		600	"	0		
B-78	48	1,990	"	0		
P-20	3	1,030	"	0		

[1] Subendocardial as well as myocardial lesions. See Figs. 5 and 8.
[2] See Fig. 1.
[3] Subepicardial as well as myocardial lesions.
[4] See Fig. 7.
[5] See Figs. 9 and 10.
[6] See Figs. 2 to 4 and 6.
[7] Subendocardial as well as myocardial lesions.

or no visible cytoplasm. Some of the nuclei were densely stained and solid; others, rather pale with the chromatin gathered into large granules. When a narrow rim of cytoplasm was visible it was pale blue. Cells were usually packed closely together in an elongated space between the muscle fibers and had in general the character of lymphocytes (Figs. 1 and 5).

The other type of lesion was made up principally of cells with large, pale, elliptical, vesicular nuclei and more or less cytoplasm,—always non-granular (Fig. 2). These cells, which have been known by various names—macrophages, endothelial leucocytes, and macrocytes—were usually less densely packed than the lymphocytes. Lesions of this type were sometimes the size of a high power field, filling the spaces between a number of muscle fibers, and occasionally were found in the vicinity of a medium sized blood vessel (Figs. 7 and 8). The cells in such lesions failed to stain deeply with pyronine in the Unna-Pappenheim stain, thus exhibiting an outspoken dissimilarity to the type cells of an Aschoff body.

While these two types of lesion were found, others were encountered that consisted of mixtures of lymphocytes and endothelial leucocytes in varying proportions (Figs. 4 and 6). Other cells were also occasionally found—mature connective tissue cells, polymorphonuclear eosinophils, polymorphonuclear neutrophils, and plasma cells (Fig. 3) and mast cells. In one rabbit a single large lesion was found containing large giant cells with many nuclei gathered together in the middle of the cells (Figs. 9 and 10).

When muscle fibers ran through a lesion they often showed loss of striation, but no other degenerative change of the parenchymatous elements was noted.

Many of the lesions have been carefully searched for microorganisms. Special stains have been employed to facilitate their demonstration,—prolonged staining with Giemsa, aqueous fuchsin, carbol-fuchsin, and gentian violet,—but no bacteria or protozoa have been seen, nor have any cell inclusion bodies been found.

DISCUSSION.

The lesions described are of interest and importance from several points of view. The rabbit is one of the most commonly used of

laboratory animals, but until recently comparatively little attention has been paid to the spontaneous diseases of this species. Mention has been made of the arterial degeneration, the nephritis, and the meningoencephalitis which previous workers have described. According to Goodpasture (11) and others the last mentioned disease is associated with a minute protozoan organism. It is probable that many other diseases are still unrecognized, and it is possible that certain experimental work on rabbits may need repetition after the spontaneous pathological conditions in this species are more clearly understood. For example, the reports of de Vecchi (12) and of Natali (13) on the production of rheumatic myocarditis in rabbits are assuredly thrown open to criticism. A comparison of their illustrations with those here presented is sufficient to make one wonder whether they observed anything except the lesions occurring in "normal" rabbits.

The work of Longcope (14) on the myocardial lesions produced by the intravenous injection of foreign proteins cannot be so easily dismissed. He describes foci in the hearts of rabbits injected with repeated doses of egg white and of horse serum strikingly similar to the lesions here described in apparently normal rabbits. In a series of controls, however, he found no pathological changes.

The incidence of the myocardial lesions in this series of "normal" rabbits (60 per cent) was higher than among rabbits included in a series of transmission experiments reported in the preceding paper (1) in which about 45 per cent showed a similar myocarditis. This higher incidence may possibly be accounted for by the fact that the present series contained older rabbits, which seem to show this myocarditis more frequently than young ones, and by the additional fact that the series extended over a longer time, as the incidence varied somewhat from year to year. It was higher, for instance, as already stated during 1920, 1921, and 1922 than during 1923 and 1924.

No etiology for the myocardial lesions has been established. It has been said that they occurred in rabbits not suffering from snuffles, coccidiosis, or hydatid disease. But nevertheless they occurred during the years when these diseases were more prevalent among our stock rabbits. Webster (15), who presents evidence indicating that

snuffles is caused by *Bacterium lepisepticum*, has found that among the stock rabbits at The Rockefeller Institute 40 per cent harbor this organism in their nasal passages without manifesting any clinical symptoms of the disease.

The myocardial lesions may be the result of mild infection, possibly with some diseases with which we are as yet unfamiliar. It is possible that they are due to a variety of causes. The diversity of the histopathological picture suggests this hypothesis.

The failure to demonstrate any microorganisms or cell inclusion bodies in the lesions does not preclude their presence at an early stage in the development of the lesions. For the cell inclusion bodies found in the lesions produced by certain filterable viruses disappear very soon after inoculation and many bacterial and protozoan organisms are destroyed by the inflammatory reaction which they incite.

SUMMARY.

The hearts of thirty-four apparently healthy adult rabbits were carefully studied microscopically. Twenty, or 60 per cent, contained lesions consisting of lymphocytes and endothelial leucocytes in varying proportions, to which were sometimes added polymorphonuclear eosinophils, plasma cells, and fibroblasts in small numbers. The lesions occurred most frequently between the muscle fibers of the papillary muscles and ventricular walls, and occasionally beneath the endocardium and epicardium. No microorganisms or cell inclusion bodies were found in the lesions.

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EXPLANATION OF PLATES.

All sections were stained with eosin and methylene blue.

PLATE 20.

FIG. 1. Lesion composed largely of lymphocytes in a papillary muscle of the left ventricle. Rabbit K-13. × 365.

FIG. 2. Left ventricle wall. Lesion composed almost entirely of endothelial leucocytes. Rabbit X-1. × 365.

FIG. 3. Large lesion close to the endocardium composed chiefly of lymphocytes with a few plasma cells. Rabbit X-1. × 365.

FIG. 4. Wall of left ventricle. Large subepicardial lesion composed of endothelial leucocytes and lymphocytes. Rabbit X-1. × 365.

FIG. 5. Small subendocardial lesion composed entirely of lymphocytes. Rabbit H-96. × 365.

FIG. 6. Interventricular septum. Large lesion composed of endothelial leucocytes and lymphocytes. Rabbit X-1. × 365.

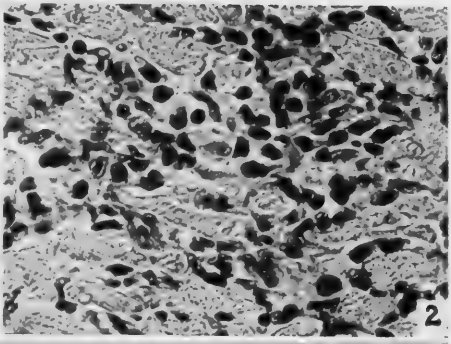
PLATE 21.

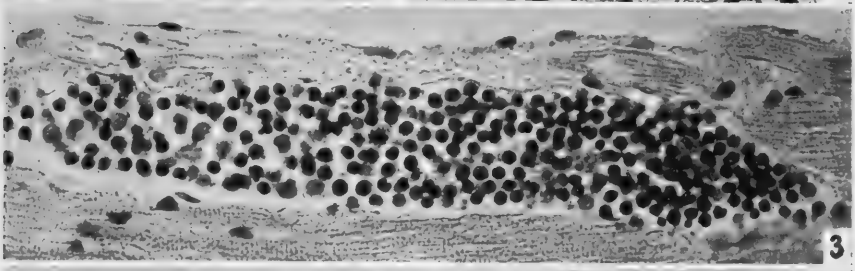
FIG. 7. Lesion composed of endothelial leucocytes and lymphocytes, adjoining a vessel. Rabbit P-608. × 365.

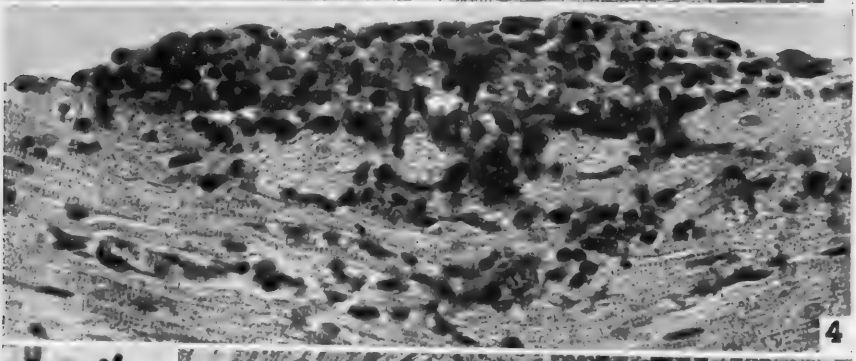
FIG. 8. Small lesion composed of endothelial leucocytes, fibroblasts, and lymphocytes, adjoining a vessel. Rabbit H-96. × 365.

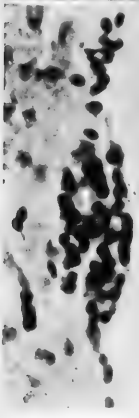
FIG. 9. Interventricular septum. Large lesion composed of endothelial leucocytes, lymphocytes, and a few fibroblasts and polymorphonuclear eosinophils. Rabbit P-653. × 365.

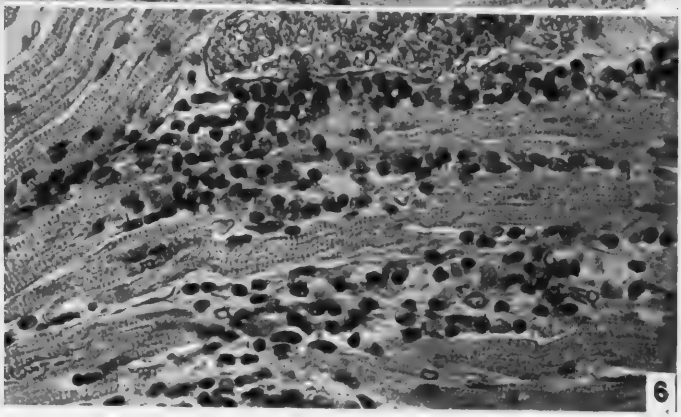
FIG. 10. Another section of the same lesion as Fig. 9 showing two large multinucleated cells. Rabbit P-653. × 365.



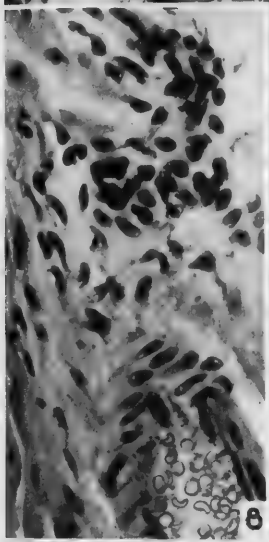


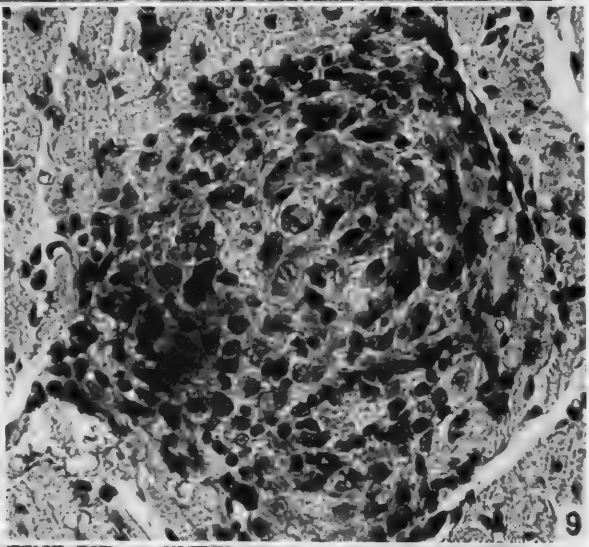


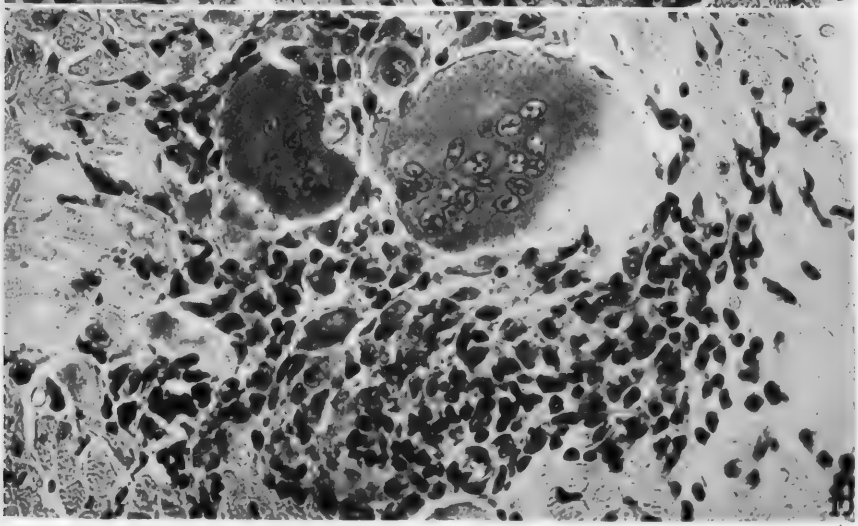




(Miller: Interstitial myocarditis in rabbits.)







(Miller: Interstitial myocarditis in rabbits.)

THE BLOOD PLATELETS IN PNEUMOCOCCUS INFECTIONS.

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The subject of the variation in the number of blood platelets, or thrombocytes, in the blood during fever and various acute infectious diseases has been studied by numerous investigators. Many different conclusions have been drawn from the observations which have been made, owing probably to the fact that many different methods have been employed in making the counts. The great number of methods and modifications of methods found in the literature indicates a general distrust of the reliability of the methods heretofore employed. Although some of the procedures give results which seem consistent enough, discrepancies occur with such frequency, without accountable reason, that suspicion is often cast on any single count. The idea is prevalent that within physiological limits, the platelets vary more in number than do the other formed elements of the blood. For instance, the normal number of platelets per c.mm. as given by different investigators varies between 200,000 and 850,000. It is probable that this conception is the result of faulty methods. Indeed our studies with an improved method which is here reported indicate that the number of platelets in the circulating blood during health is fairly constant.

Owing to the absence of any consensus of opinion concerning the number of platelets which may be considered normal, the interpretation of variations in the counts during disease has been difficult. In general, however, it has been fairly well established that during the course of the acute infectious diseases the number of platelets in the blood is decreased, and that during convalescence the number becomes normal or even greater than normal. As regards the platelets in the blood during pneumonia, especially in lobar pneumonia associated with the pneumococcus, only a few studies have been made.

No investigators have reported the results of frequent observations in individual cases and, consequently, no information is at hand regarding the changes which occur during the various stages of the disease.

It, therefore, seemed of value to make a series of observations regarding the platelets in pneumonia, with a method giving consistent findings, and the present paper states the results of these observations.

Method.

In most of the older methods for counting platelets, the blood was obtained by pricking the finger, smears were made, the relative number of platelets and erythrocytes was determined and the absolute number of platelets was then calculated. The total number of red cells per c.mm. of blood was found by the usual method.

Possibly the greatest error in most of this previous work resulted from the faulty method of obtaining the blood. When blood is allowed to come into contact with tissue juices, clumping of the platelets to a greater or lesser degree almost always occurs. Therefore, if the specimen for examination is obtained by pricking the skin, under which condition contamination with tissue juices always occurs, one cannot expect to obtain correct or consistent counts. Moreover, when the attempt is made to estimate the relative number of platelets and red blood cells in the same microscopic preparation, frequently many platelets are covered by red cells and faulty estimations are obtained.

In 1919 Oluf Thomsen (1) devised a method in which the platelets are counted directly and this method we believe to be more reliable than those previously employed. He made use of a well known fact; namely, that if blood be mixed with a solution of sodium citrate to prevent clotting, and this mixture be allowed to stand until the red and white cells have settled to the bottom, the platelets remain for a long time uniformly suspended in the supernatant fluid. The platelets in this suspension may then be counted directly and, since the dilution employed is known, the number of platelets per c.mm. of undiluted blood may be readily estimated. This method has been employed by Gram (2) by Schenk and Spitz (3) and I (4) have also found it satisfactory in previous studies. Briefly the technique as described by Thomsen is as follows:

Blood is withdrawn from the vein and run into a graduated centrifuge tube containing 0.5 cc. of 10 per cent solution of sodium citrate until the 5 cc. mark is reached. The tube is inverted several times to facilitate thorough mixing. The tube is then allowed to stand until the red and white blood cells have settled to the bottom. The supernatant fluid containing the platelets in suspension is then diluted 1:20 in a leucocyte-counting pipette by means of a solution containing sodium chloride, formaldehyde, and brilliant cresyl blue, and the platelets are counted in a hemocytometer chamber. The number of platelets per c.mm. of

blood can then be readily calculated; the volume of the red cells is taken into account, being determined either directly or by the method of calculation from the hemoglobin content as employed by Gram (2).

Certain modifications in technique were made which render the method more simple without detracting from its accuracy. The following is a description of the technique I have employed.

0.9 cc. of blood is drawn from the vein into a tuberculin syringe containing 0.1 cc. of a 10 per cent solution of sodium citrate. It is important to employ a needle that is not too small; a No. 20 Luer needle is most satisfactory. After removing the needle the blood is drawn into the upper part of the barrel, the finger-tip is placed over the open end and the syringe is inverted several times to insure thorough mixing. Then an inch or more of rubber tubing is slipped over the open end, and the tubing bent over and laid back on the barrel of the syringe to which it is firmly attached by wrapping with a rubber band. The piston is then removed and the syringe placed in a vertical position to permit sedimentation. After this has occurred a dilution of 1:20 of the supernatant fluid is made in a blood-counting pipette with normal saline as a diluting fluid. A drop from the diluting pipette is placed on the hemocytometer slide and the platelets allowed to settle for about 15 minutes. The platelets may then be counted with as little difficulty as are erythrocytes, in the usual blood-counting technique. The platelets seen in 80 small squares are counted. Three ciphers are added to the number obtained and this gives the number of platelets per c.mm. of the citrated plasma. The syringe containing the citrated blood is then centrifuged for 20 minutes at 2,000 revolutions per minute. By means of the following proportion the number of platelets per c.mm. of citrated blood is calculated.

No. of platelets per c.mm. of citrated plasma	:	x	: :	Total amount of the mixture	:	Amount of citrated plasma

(x equals the number of platelets per c.mm. of citrated blood.) Then, if 10 per cent of this number is added on account of the original dilution with citrate, the result gives the approximate number of platelets per c.mm. of whole blood.

By the above method, the average number of platelets per c.mm. of normal human blood has been found to be 350,000, varying slightly from time to time in the same individual. In counting the blood platelets in rabbits it was found impossible to enter the ear vein with a needle which was large enough to obviate clumping of the platelets. Therefore the skin over the vein was smeared with vaseline, and the vein was cut across. As the blood flowed out rapidly, it was allowed to drop into the syringe, the mouth of which was also coated with vaseline. In spite of the precautions observed, clumping of the platelets was occasionally encountered, necessitating a repetition of the procedure. The number of platelets in normal rabbit blood was found to be considerably higher than that in normal human blood, from 500,000 to 600,000.

Platelets in Pneumococcus Infections.

There will be reported the fluctuations in the number of platelets in thirteen cases of lobar pneumonia, in three cases of bronchopneumonia, and in two rabbits in which pneumococcus septicemia was produced. All counts were made by Thomsen's method, with the modifications previously mentioned. As a rule, counts were made daily, or every other day during the 1st week; thereafter, counts were made at longer intervals. The charts are arranged so that the heavy line represents an arbitrary normal level of rectal temperature, normal number of leucocytes per c.mm., and normal number of platelets per c.mm. in the blood.

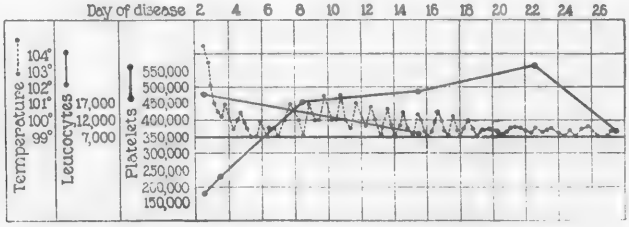
In all of the uncomplicated cases of lobar pneumonia with recovery, the curves made by charting the various counts are of similar contour. In each instance a thrombopenia of from 125,000 to 243,000 platelets per c.mm. occurred during the fever, and a subsequent thrombocytosis was observed after the temperature had reached normal. Usually the number did not begin to increase until several hours after the crisis had begun. Frequently, the increase commenced only after the temperature had been normal for several hours. In the subsequent 6 to 9 days, the number of platelets increased steadily until the normal limit was greatly exceeded. The greatest number observed was 748,000 per c.mm. After the highest point in the curve had been reached, the number again diminished until the normal level was regained in about the same number of days.

It is of interest to note that in Case 1, in which fever persisted after the crisis, the rate of increase in the number of platelets was somewhat retarded, and that in Cases 1 and 2 the usual high number was not attained. Cases 2 and 3 are also of interest since a secondary rise in temperature, due to severe serum disease in Case 3 and to pleurisy in Case 2 was in each instance accompanied by a second diminution and subsequent increase in the number of platelets. Text-fig. 4 shows an example of the usual curve obtained.

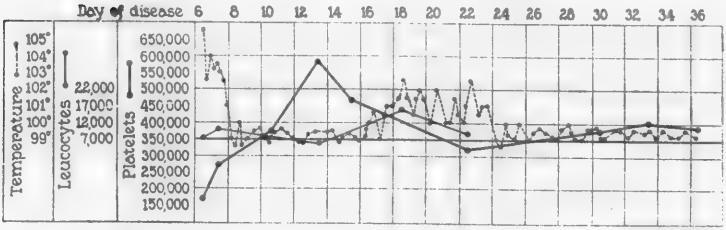
In one case the first count was made 22 hours after the initial chill and the number of platelets was then still within the normal limit. The diminution in the number of platelets appeared later. Judging from this and from observations on rabbits, it seems likely

that the diminution in the number of platelets occurs only after the infection is well established.

Two cases of lobar pneumonia which ended fatally were studied and in these no diminution in the number of platelets was observed. Both of these cases were admitted into the Hospital late in the disease and in both a pneumococcus septicemia was present. In one of these cases admitted on the 5th day of illness, the platelet count showed a tendency to increase, being 354,000, 386,000, and 437,000 on 3 successive days. Unfortunately we have no way of knowing if a thrombopenia preceded this rise or not.



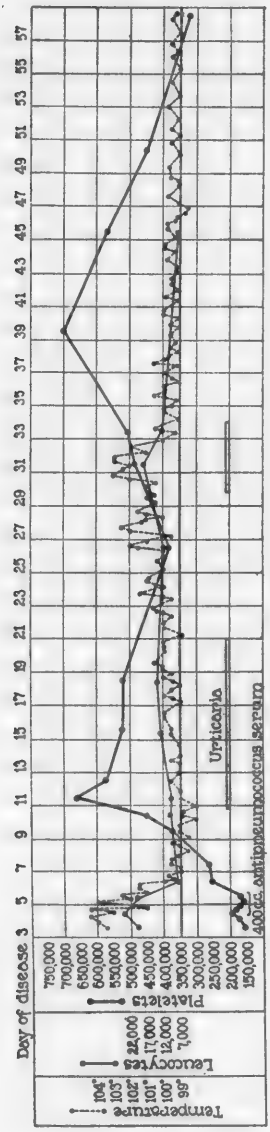
TEXT-FIG. 1. Case 1. Type IV lobar pneumonia.



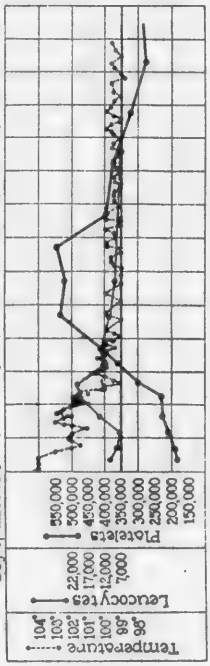
TEXT-FIG. 2. Case 2. Type IV lobar pneumonia; pleurisy.

In addition to the studies made on patients with lobar pneumonia, three patients with bronchopneumonia were studied, two mild cases and one ending in death. In one of the mild cases the number of platelets found was normal; in the other one a fluctuation in the number from 160,000 to 360,000 was observed. In the fatal case counts of 90,000 and 70,000 platelets per c.mm. were obtained.

One experimental rabbit received an overwhelming dose of pneumococci and the changes in the platelets were studied. 24 hours after

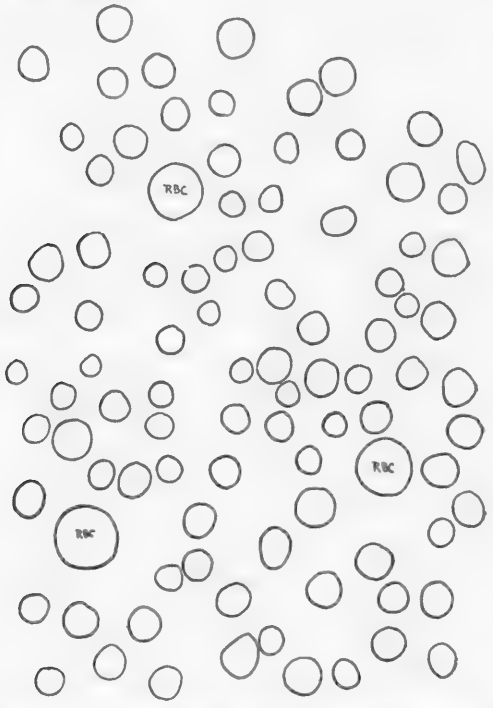


TEXT-FIG. 3. Case 3. Type I lobar pneumonia; serum sickness.



TEXT-FIG. 4. Type II lobar pneumonia.

infection there was found a diminution in the number of platelets. The number then further decreased rapidly until death occurred. In a second animal, which received a much smaller dose, only a very slight diminution in the number of platelets was observed, and a postfebrile rise occurred.



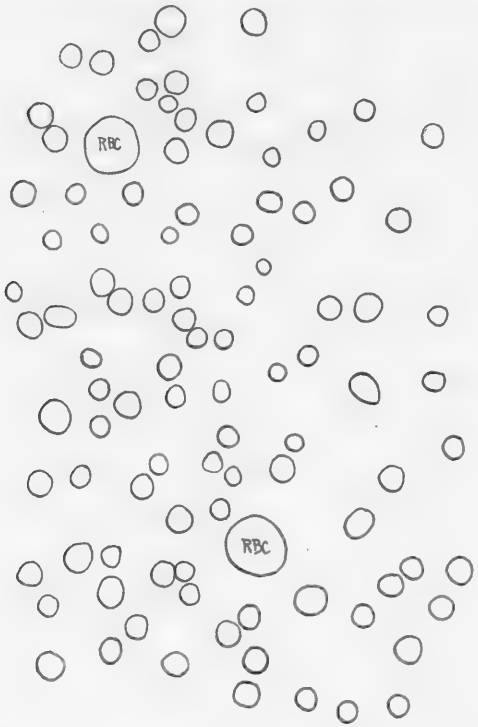
TEXT-FIG. 5. Normal blood platelets. 350,000 per c.mm.

Morphology of the Platelets.

A study was made of the morphology of the platelets at various times during the course of pneumonia and also during the postfebrile period. It is generally conceded that no nucleus is present in platelets and that normal intact platelets are round or oval discoid bodies consisting of a perfectly homogeneous substance, and that the granules

become visible in the platelets only after the death of the cell. According to various authorities normal blood platelets vary from 2 to 7 microns in diameter. Webster (5) states that the size varies inversely with the number.

I have made observations on the variations in size of platelets.



TEXT-FIG. 6. 2nd day of disease. Platelets 123,000 per c.mm.

For this purpose I have taken tracings from stained smears by means of a camera lucida. Smears were made during the course of illness when the count was lowest, during the stage of so called regeneration, when the count was above normal, and again after the number had returned to normal. Tracings were made from each smear and compared. It is realized that with this method the platelets were observed when spread out on a slide; that is to say, not in their normal condition. However, no other satisfactory method is available.

The attempt was made to stain all films to approximately the same intensity by allowing 12 drops of Wright's stain to remain on the slide for 30 seconds, followed by 9 drops of distilled water, the mixture being allowed to stand for 2½ minutes and then washed off with distilled water. Even with this constant technique, considerable differences in intensity in depth of staining were encountered in different smears, probably because the films were of different thickness, or owing to the different physical characteristics of the films, the platelets, or their surrounding matrix.

TABLE I.

Differential Count of Platelets.

Case No.	Day of disease.	Large, many granules.	Small, many granules.	Large, few granules.	Small, few granules.	Large platelets.	Small platelets.	Platelets with many granules.	Platelets with few granules.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2	6	18	58	9	15	27	73	76	24
	13	19	30	37	14	56	44	49	51
4	3	4	45	18	33	22	78	49	51
	9	14	31	26	29	40	60	45	55
7	3	4	51	25	20	29	71	55	45
	5	10	51	9	30	19	81	61	39
	14	2	10	8	80	10	90	12	88
	27	10	33	22	35	32	68	43	57
8	4	26	64	4	6	30	70	90	10
	9	12	28	31	29	43	57	40	60
9	2	8	69	7	16	15	85	77	23
	8	12	46	30	12	42	58	58	42
	27	15	41	18	26	33	67	56	44
Normal individual.		9	20	36	35	45	55	29	71
" "		21	25	34	20	55	45	46	54

The platelets were studied in this way in ten cases and in all cases it was found that the average size of the platelets was distinctly smaller during the thrombopenic period than when the count was increased or normal, as shown by the accompanying figures. In two of the cases the preponderance of the smaller size platelets persisted during the stage of thrombocytosis. In the other cases, during the period

of thrombocytosis, the average size of the platelets was that seen in normal individuals. In no instance, either during the stage of thrombocytosis or during that of thrombopenia, were the platelets very large. The largest one found on measuring 2,500 had a diameter of 6 microns. The studies of normal blood showed that the platelets vary in size between 2 and 5 microns.

An attempt was made to classify the platelets by a differential count in a number of instances. A very arbitrary classification was devised; namely, large with many granules, large with few granules, small with many granules, small with few granules. Roughly, the platelets over 3 microns in diameter were considered "large," and those under 3 microns were considered "small." Obviously, differences in intensity of staining affected the classification considerably. However, even with this crude method it is evident from Table I that a distinct preponderance of the so called well filled forms, or the forms containing many granules, occurred during the febrile period of pneumonia. This observation is in agreement with that of Zeller (6) who found platelets to be fewer and more granular during acute diseases.

DISCUSSION.

Relation of Platelets to Immunity.

It has been suggested upon numerous occasions that the blood platelets play a part in the defense of the body against bacterial invasion.

Govaerts (7) states that the platelets attach themselves to foreign bodies, such as bacteria, in the blood stream, and influence their destruction. Delrez and Govaerts (8) injected staphylococci and paratyphoid bacilli intravenously into rabbits and observed that the platelets soon attach themselves in large numbers to the bacteria and that the bacteria then disappear from view. But if instead of staphylococci and paratyphoid bacilli, pneumococci were injected into rabbits no agglutination with the platelets occurred, the number of colonies in the blood culture was not diminished, and the bacteria did not disappear from the blood. If the rabbit was then injected with antipneumococcus serum, clumping of the pneumococci occurred without association of the platelets and the bacteria disappeared from the blood.

Later, Bull and McKee (9) demonstrated that pneumococci circulating in an actively immunized rabbit, whose platelets have been removed from the circula-

tion by treatment with antiplatelet serum, agglutinate just as quickly and completely as they do in a rabbit which has the normal number of platelets. They conclude that the association of platelets with bacterial clumps is merely incidental.

Cramer and Drew (10) observed that when animals are raised in a dark room a thrombopenia occurs and that such animals are very liable to infections. After exposure to a mercury vapor lamp, there is an increase in the number of platelets and a decrease in the liability to infection. Similarly, Cramer, Drew, and Mottram (11) found that a withdrawal of soluble vitamine A from the diet of a rat also leads to a thrombopenia and a diminished resistance to infection, and that in animals treated in this way infections often develop spontaneously. When soluble vitamine A is again added to the diet, the platelets increase in number and the animals recover from the infections. They conclude from these experiments that the blood platelets represent a defensive mechanism against bacterial invasion. Bedson and Zilva (12) have been unable to confirm these observations. It would seem that the mere absence of light and withdrawal of an important factor of the diet may alone be sufficient to render the animals more liable to infection irrespective of platelet changes, and that this tendency may disappear upon reestablishment of normal conditions.

The observations on patients with pneumonia suggest that the diminution in the number of platelets is a result of the infection or the attendant fever rather than that thrombopenia is a factor in the occurrence of infection. These observations, moreover, do not indicate that the platelets play an important part in recovery from infection.

Relation of Platelets to the Coagulation Time of the Blood during Pneumonia.

In connection with the observations on the behavior of the platelet count, it is interesting to correlate them with the work of Dochez (13) and Minot and Lee (14), who showed that the coagulation time in pneumonia is lengthened, but tends to become shorter about the time of crisis. The blood used in their determinations was obtained by venepuncture. Anders and Meeker (15) state that the coagulation time is somewhat shortened during pneumonia. The contradictory findings are probably explained by the fact that these authors obtained the blood for their observations by pricking the skin. The admixture of "tissue juices" containing cytozyme or thrombogen which is rendered inevitable by this procedure vitiates the accuracy of the determination of the coagulation time.

It is highly probable that the diminution in the number of platelets during pneumonia plays an important rôle in the delayed coagulation time. Bordet (16) and others have shown that the platelets participate actively in the production of the coagulating principle. Minot and Lee (14), Duke (17), Lesourd and Pagniez (18) state that the clot does not retract, or retracts poorly, when the platelets are diminished in number. This phenomenon was observed in the blood during the thrombopenic stage in the cases of pneumonia reported in this investigation.

Behavior of the Platelets after Injection of Foreign Substances.

Numerous observers report that the parenteral injection of foreign substances is followed by a diminution in the number of platelets in the blood. For instance, Achard and Aynaud observed a diminution in number following the injection of albumin, Bedson one after agar serum, Zeller and Schiff and Matyas one after serum and milk, and Pickering and Hewitt a like phenomenon after the injection of nucleic acid. However, in the five cases observed in this study, which received repeated injections of 100 cc. doses of antipneumococcus (horse) serum, no immediate effect on the number of platelets was observed. In several instances counts were made immediately before, immediately after, and again 6 hours after the injection of the serum. Practically no changes in the platelet counts which could be attributed to the injection of the serum occurred. It is possible that the injection of foreign substances into normal individuals produces a fever with the usual concomitant reduction of the number of platelets.

SUMMARY.

1. A study was made of the behavior of the blood platelets during the course of pneumonia in man, as also in several rabbits experimentally infected with pneumococci.

2. The number of platelets begins to diminish after the infection has become established. A thrombopenia occurs during the febrile period. Immediately following the onset of the crisis in lobar pneumonia or after the temperature begins to fall by lysis, the platelets begin gradually to increase in number. In the postfebrile period, the

platelet count increases until the normal number is greatly exceeded, and there is a return to the normal number only after about 2 weeks.

3. Sequelæ, characterized by fever, following pneumonia are accompanied by a renewed diminution in the number of platelets. After subsidence of the temperature their number usually increases until the normal is again exceeded.

4. The platelets in general are smaller and contain more granules during the thrombopenic stage of pneumonia.

5. Intravenous injection of antipneumococcus serum during pneumonia ordinarily produces no variation in the number of platelets.

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PRODUCTION OF IMMUNITY IN MICE BY INHALATION OF PNEUMOCOCCI.

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In preceding papers (1) it has been shown that following inhalation of virulent pneumococci they generally disappear from the lungs of normal mice within a few hours, that infection rarely occurs, and that in only a few instances does death result from a septicemia. The regularity with which pneumococci may be recovered from small pieces of lung is evidence that the organisms enter the lower respiratory tract. Despite the presence of virulent organisms deep in the respiratory tract the animals are only infrequently infected, as evidenced by the large number of survivals. Furthermore, since the organisms are only found in the lungs for a few hours after inhalation there must be an efficient method for their disposal. If mice are first intoxicated with alcohol and then sprayed with pneumococci by the same technique, the pneumococci persist in the lungs for much longer periods and fatal septicemia occurs in as many as 40 per cent of the mice. Evidently alcohol interferes in some way with the mechanism concerned with the disposal of inspired bacteria. No evidence was obtained, however, as to the exact method of this disposal. If the removal of the bacteria is accomplished solely by the action of the ciliary currents, *i.e.* mechanically, one would suspect that no immunity would be acquired, as the respiratory tract is really "outside the body." If, on the other hand, the inspired bacteria penetrate the surface epithelium and actually gain access to the lung tissue proper, some degree of immunity might reasonably be expected to develop.

It is the object of this paper to report certain experiments which show that immunity is present in normal mice which have been repeatedly exposed to an atmosphere containing live virulent pneumo-

cocci in suspension, and to a less extent in mice which have been exposed to an atmosphere containing heat-killed organisms, and further in mice which have survived one or more exposures to a spray of virulent pneumococci while intoxicated.

Method.

The mice were exposed to the living or dead bacteria in the spray chamber previously described (1). The animals were sprayed repeatedly at intervals of from 2 to 3 days with 50 cc. of (1) an 18 hour broth culture of the living virulent organisms, or (2) similar cultures killed by heating at 60°C. for 30 minutes, or (3) washed heat-killed organisms suspended in salt solution.

In the case of intoxicated mice, the alcohol was administered intraperitoneally in 10 per cent solution in saline, 1.5 cc. being given 1 hour before spraying.

The degree of immunity acquired by the mice was measured by the ability of the animal to withstand an intraperitoneal injection of a fatal dose of virulent organisms. These tests were performed 10 days after the last spraying. The survival of mice following an intraperitoneal injection of dilutions of 0.0001, 0.00001, and 0.000001 of an 18 hour broth culture of the homologous organism was used as a measure of the degree of immunity developed in the mice. The virulence of the strain of Type I pneumococcus used throughout was such that a millionth cc. injected intraperitoneally invariably resulted in death in 48 hours. Therefore, the survival of any of the treated mice for a period of 5 days following injection was accepted as proof of an acquired immunity.

EXPERIMENTAL.

It is necessary to emphasize the fact that although the pneumococcus is an organism which generally disappears from the lungs of normal mice within a few hours following inhalation, rarely giving rise to infection, yet, during the course of repeated exposures a fatal invasion of the blood stream will occur in a certain number of mice. In other words, organisms may penetrate the tissues in numbers sufficiently large to produce a fatal infection. Table I shows the number of normal animals which succumbed during the course of spraying with virulent pneumococci.

From the table it is seen that only twenty-nine deaths occurred among 449 normal mice which were repeatedly exposed from one to ten times in gradually decreasing numbers to an atmosphere containing virulent pneumococci in suspension. Unfortunately nine of the animals dying were obtained in a state of postmortem decomposition

such that cultures from them would have been useless, but pneumococci were recovered from the heart's blood of fourteen. It is not possible to state definitely at which exposure infection took place, but in the light of subsequent work it is probable that it occurred at the spray immediately preceding death. The table also brings out the interesting fact that the greatest number of deaths occurred after the second exposure, and that the mortality tended to lessen, until after the tenth exposure no animals succumbed. In other words, those mice which were most susceptible to pneumococcus died early. As will be shown, the surviving mice were those which not

TABLE I.

Fate of Mice Exposed to from One to Ten Inhalations of Pneumococcus Cultures.*

No. of times exposed to virulent pneumococci.	No. of mice exposed.	No. dying.	Pneumococcus recovered from heart's blood.	Pneumococcus not recovered from heart's blood.
1	449	—	—	—
2	446	10	7	3
3	337	4	1	3
4	312	8	4	4
5	211	2	—	2
6	210	1	—	1
7	167	1	1	—
8	165	2	—	2
9	109	1	1	—
10	108	—	—	—
	449	29	14	15

* The discrepancy in the total numbers is due to the fact that some animals were used for intraperitoneal tests or for other experiments.

only had the greatest natural resistance but also had acquired a certain degree of immunity as a result of repeated exposures to the pneumococcus atmosphere.

To demonstrate that a degree of immunity is acquired by mice, even after the second exposure to virulent pneumococci, the following experiment was performed which will serve as an example of other similar ones. 10 days after the second of two exposures to virulent pneumococci the immunity of thirty mice was tested by an intraperitoneal injection of virulent organisms. The results are given in Table II.

From Table II it is seen that six of the thirty animals which had been twice exposed survived an intraperitoneal inoculation of pneumococci greatly in excess of that amount which invariably proved fatal in normal controls. This experiment demonstrates that the resistance of certain animals was increased, although there is a considerable individual variation in this increase.

The degree of immunity acquired by mice which had been exposed more frequently, up to ten times, to an atmosphere containing virulent

TABLE II.

Virulence Test on Thirty Mice Twice Previously Exposed to a Pneumococcus Spray.

Pneumococcus Type I. 0.0001 cc.	0.00001 cc.	0.000001 cc.	Normal controls 0.0001 to 0.000001 cc.
D. 34 hrs.	D. 24 hrs.	D. 28 hrs.	
" 36 "	" 24 "	" 34 "	
" 36 "	" 36 "	" 36 "	
" 36 "	" 48 "	" 36 "	
" 36 "	" 48 "	" 36 "	
" 40 "	" 48 "	" 44 "	D. within 48 hrs.
" 40 "	" 48 "	" 44 "	
" 40 "	" 72 "	S.	
" 44 "	S.	"	
S.	"	"	
Died. 9	8	7	
Survived. 1	2	3	

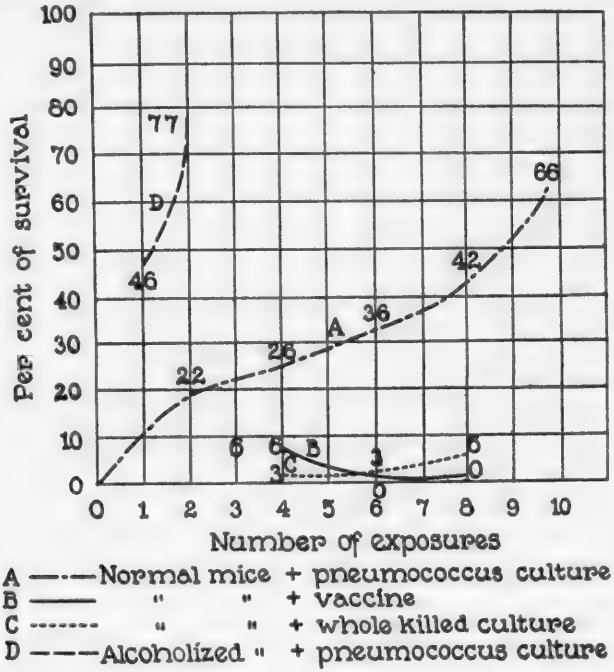
D. indicates died.

S. indicates survival.

The figures represent the number of hours elapsing before the death of the animal.

pneumococci has been tested in like manner. Ten mice in each series were injected intraperitoneally with 0.0001, 0.00001, and 0.000001 cc. of a virulent pneumococcus culture, respectively. The results of these experiments, including the one tabulated above, are given in Text-fig. 1. In this figure the dot and dash line (*A*) represents the results following exposure to virulent living organisms. It is seen that after two exposures 22 per cent of the total number of mice survive an intraperitoneal injection of virulent organisms, while after ten exposures 66 per cent survive.

Since the mice which were naturally of high susceptibility to pneumococcus infection had died early in the course of the immunization, these experiments were performed on mice which possessed a certain natural resistance to the pneumococcus. However, the figures indicate a greater degree of immunity than can be accounted for by mere "selection of the fittest," as is sufficiently evident from the failure


Per cent of survival
Number of exposures
A —·—Normal mice + pneumococcus culture
B ——— " " + vaccine
C ------- " " + whole killed culture
D ———Alcoholized " + pneumococcus culture

TEXT-FIG. 1.

of any controls to survive (Table II). Furthermore, the gradual increase of acquired protection clearly indicates that repeated exposures to the pneumococcus induce a progressively increasing active immunity. It is thus evident that following inhalation of virulent pneumococci a definite protection is afforded mice even on intraperitoneal inoculation and that the amount of this protection depends to a considerable degree on the number of previous exposures.

In order to determine whether any immunity was produced by the inhalation of dead organisms, mice were sprayed from four to eight times with either whole killed cultures of pneumococcus or dead organisms suspended in saline. The degree of active immunity developed was measured by the intraperitoneal injection of virulent organisms. The results are charted in Text-fig. 1, the dotted line (*C*) representing the results with whole killed cultures and the unbroken line (*B*) those with dead organisms suspended in salt solution. It is seen that exposure to killed organisms likewise produces protection to the intraperitoneal injection of virulent organisms, though one relatively slight as compared with the resistance obtained when living organisms are inspired.

Finally, the degree of acquired immunity in mice which had recovered from the combined administration of alcohol intraperitoneally and pneumococcus spraying was determined. The protection tests in these animals are represented in Text-fig. 1 by a broken line (*D*). The animals which had been exposed to a pneumococcus spray while intoxicated showed a decided immunity which increased in proportion to the number of exposures. Of the mice which had survived one or two preliminary treatments with alcohol and pneumococci, 46 and 77 per cent, respectively, were sufficiently immune to overcome an intraperitoneal injection of virulent organisms. While in this particular instance the increase of immunity is more prompt and slightly greater than in the non-alcoholized animals, the number of mice which survive the treatment itself is very much less and the incidence of infection far greater. Consequently, the animals which successfully survive the preliminary treatment may be considered to possess a high natural resistance. However, the great degree of immunity exhibited by these mice can hardly be accounted for by the fact that the more susceptible had died as a result of exposure to a pneumococcus atmosphere while intoxicated. Indeed, since the pneumococci persist much longer in the lungs of intoxicated mice, it seems not unlikely that the noteworthy increase in the immunity of the animals is due to a greater and more rapid development of antibodies.

DISCUSSION.

The results obtained by Webster (2) on microbic virulence and host susceptibility in mouse typhoid infection are strikingly paralleled by the results of the present work with pneumococci. This author found that if he fed 100 mice 6,000,000 typhoid bacilli by stomach tube, approximately 70 died with the organism in the blood and stools, and 30 survived. In 20 of the latter typhoid bacilli were present in cultures from the stools only during the first few days and not in cultures from the blood,—in which respect these animals were naturally immune,—while in the remaining 10 animals the organisms persisted in the stool sometimes as long as 6 months and were temporarily present in the blood stream. Such animals recovered and developed agglutinins. The second group mentioned represent animals which develop an acquired immunity after passing through the so called "carrier state" or condition of "latent infection."

Cecil and Steffen (3) were able to show that intratracheal injections of killed cultures rendered monkeys insusceptible to later intratracheal injections of living pneumococci. They were unable, however, to produce any immunity by spraying killed cultures into the pharynx. Although Jones (4) could immunize rabbits against *Bacillus avisepticus* by the intratracheal injection of killed cultures, he was unable by means of sprays of heat-killed organisms to increase the resistance appreciably. He concluded that in the latter instance insufficient antigen was absorbed to afford measurable protection against subsequent intratracheal infection.

From the foregoing experiments it appears that a high degree of active immunity is produced in mice following repeated inhalations of living pneumococci, and that a less marked increase in resistance is afforded animals exposed to a spray of dead organisms. To explain this acquired protection, one is forced to assume that the inhaled bacteria or bacterial cells actually gain access to the body tissue; that is, they are not only implanted on the mucosa of the lower respiratory tract, but actually penetrate the respiratory epithelium. Very few living organisms probably gain entrance at any one time. In fact, I have never observed pneumococci in histological sections from the animals, while even culturally it is not sufficient merely to

inoculate a blood agar plate with the cut surface of the lung tissue, for so few are the organisms present that sections of the tissue itself must be cultured in broth. Even under these optimum conditions growth is not infrequently delayed until after 24 hours. One may suppose the high degree of active immunity which results from the inhalation of live organisms to be due to the limited multiplication of the organisms within the tissues. The possibility exists, however, that actual multiplication of the pneumococci does not occur but that mice may be immunized to a fairly high degree by the repeated penetration of a very few virulent organisms which are quickly destroyed.

CONCLUSIONS.

1. A definite degree of active immunity can be induced in mice through the repeated inhalation of live pneumococci.

2. Only slight immunity is induced in mice by the repeated inhalation of killed organisms.

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